Molecular
Cloning

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Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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Molecular
Cloning
A LABORATORY MANUAL
SECOND EDITION

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Preface to First Edition

This manual began as a collection of laboratory protocols that were used during the 1980 Cold Spring Harbor course on the Molecular Cloning of Eukaryotic Genes. These procedures had been in use in our laboratories at that time but were scattered throughout the notebooks of many different people. In 1981 we decided to produce a more complete and up-to-date manual not only for use in the next Cold Spring Harbor course, but also for eventual publication. Out of the many permutations of the methods being used, we assembled a set of "consensus protocols," which were photocopied and widely distributed to many laboratories even as the 1981 course was underway. Then in the winter of 1981–1982, the manual was substantially rewritten, and new or revised protocols and figures, as well as entirely new chapters, were added.

Even since this last rewriting, however, the field has progressed: New methods are constantly being invented and existing techniques are altered in response to changing needs. Although we have included in this manual only those protocols that have been thoroughly tested and used successfully in our laboratories, we make no claim that they are inviolable or perfect. We would welcome suggestions for improvements, and we would be grateful to be told about any new procedures that are devised.

The evolution of protocols poses the difficult problem of attribution. We have tried to give credit at appropriate places in the text to the people who originally developed the procedures presented here, but in many cases tracing a particular method to its undisputed roots has proved to be impossible. We therefore wish to apologize—and to express gratitude—to those we have been unable to acknowledge for an idea, procedure, or recipe. Our major function has been to compile, to verify, and, we hope, to clarify, less frequently we have introduced modifications, and only in rare instances have we devised new protocols. In large part, then, the manual is based on procedures developed by others, and it is to them that any credit belongs.

Because the manual was originally written to serve as a guide to those who had little experience in molecular cloning, it contains much basic material. However, the current version also deals in detail with almost every laboratory task currently used in molecular cloning. We therefore hope that newcomers to cloning and veterans alike will find material of value in this book.

Although molecular cloning seems straightforward on paper, it is more difficult to put into practice. Most protocols involve a large number of individual steps and a problem with any one of them can lead the experimenter into difficulty. It is a good idea to verify the products of each step and to include controls to check the efficiency of each reaction. To deal with these problems, a well-founded understanding of the principles underlying each procedure is essential. We have therefore provided background information and references that may be useful should trouble occur.

This manual could not have been written without the help and advice of members of our laboratories and contributions from many others. We therefore wish to thank Joan Brooks, John Fiddes, Mary-Jane Gething, Tom Gingeras, David Goldberg, Steve Hughes, David Ish-Horowicz, Mike Mathews, Patty Reichel, Joe Sorge, Jim Stringer, Richard Treisman, and Nigel Whittle. We wish particularly to thank Arg Efstratiadis for his helpful discussions and criticisms of Chapter 7; Brian Seed for permission to include a description of his unpublished procedure for screening libraries by recombination (Chapter 10) and many other useful suggestions; Doug Hanahan for advice on transformation (Chapter 8); Bryan Roberts for suggestions on methods of hybrid-selection and cDNA cloning, Doug Melton for providing a protocol for injection of *Xenopus* oocytes; Ronni Greene for suggesting improvements to many protocols; Nina Irwin for providing a critical anthology of methods available for expressing eukaryotic proteins in bacteria (Chapter 12), Rich Roberts for supplying the computer analysis of the sequence of pBR322, Barbara Bachmann and Ahmad Bukhari for reviewing and correcting the list of *E. coli* strains; and Tom Broker, Louise Chow, Jeff Engler, and Jim Garrels for producing the elegant photographs used for the front and back covers.

We also thank all those who participated in the Cold Spring Harbor Molecular Cloning courses of 1980 and 1981. They were an excellent group of students, who struggled through the first two drafts of the manual and made many useful suggestions. We also thank Nancy Hopkins, who helped us to teach the course the first year and convinced us that producing a manual would be a worthwhile task. In 1981 Doug Engel helped teach the course and suggested many improvements to the manual. Contributing to the success of both courses were the efforts of the teaching assistants, who were Catherine O'Connell and Helen Doris Keller in the summer of 1980 and Susan Vande-Woude, Paul Bates, and Michael Weiss in 1981.

We wish to thank Patti Barkley and Marilyn Goodwin for their cheerfulness and forbearance during the typing of successive revisions of the manuscript. Our artists, Fran Cefalu and Mike Ockler, worked with great dedication and perseverance to produce the drawings for the manual. Joan Ebert kept track of the many references added to and deleted from the text and assembled the reference list. We are also grateful to Nancy Ford, Director of Publications, Cold Spring Harbor Laboratory, for her encouragement and support. Finally, without the patience, skill, and diplomacy of Doug Owen, who prepared the manuscript for the printer and helped us in many other ways, this book would not exist.

Tom Maniatis
Ed Fritsch
Joe Sambrook

Preface to Second Edition

Since the publication of the first edition of this Laboratory Manual in 1982, there has been a vast increase both in the number of people who use molecular cloning and in the range and power of the techniques used to handle recombinant DNA. This remarkable proliferation of cloning methods is reflected in the number of gene sequences in the GenBank DNA sequence data base. In 1982 there were fewer than 350 gene sequences on file, but in less than four years this number grew to almost 5000. Today over 15,000 sequences are listed. These figures, impressive as they are, do not do justice to the increased sophistication with which cloned genes are now analyzed. In 1982, respectable journals would still accept manuscripts containing little more than the partial sequence of a cDNA clone; today, the publication of a complete sequence is taken for granted, and papers describing the initial cloning of a cDNA will often also contain elegant accounts of the expression of the gene product in prokaryotic or eukaryotic hosts. In most cases, this first paper is rapidly followed by others that use site-directed mutagenesis to explore the relationship between the structure and function of the relevant protein. The cloning and analysis of elements that regulate the expression of eukaryotic genes has moved at a similar, exciting pace.

This increase in the range and speed of molecular cloning is reflected in the tripling of size of this manual and its consequent division into three volumes. Techniques that were mentioned only in passing in the first edition, such as mutagenesis, expression of cloned genes in mammalian cells, and dideoxy-mediated sequencing, are now described in depth; new sections have been added that deal with recently invented techniques, such as amplification of DNA by the polymerase chain reaction; and we have included modern variations and embellishments of many of the basic methods that were the mainstay of the first edition. We hope that any inconvenience caused by the expansion in size of the manual will be compensated by an increase in the richness of its content. We hope that this second edition will be a resource for the experienced cloner, a starting point for the student, and a guidebook for the next generation of investigators in molecular cloning.

The burgeoning of molecular cloning has also led to its commercialization, and we are now able to pruchase a wide variety of high quality and reasonably priced reagents and enzymes. Although this is a very positive development, it has had a few unfortunate side effects. One is the proliferation of preassembled kits to carry out particular cloning tasks. Although these kits reduce the possibility of trivial errors, they also tend to discourage experimenters from thinking about what they are doing. It becomes all too easy to follow blindly instructions to add 2 μl of solution A without knowing what the particular reagent is, why it is necessary, or why it is added at a particular point in the protocol Kits therefore reward the scientifically illiterate and inhibit the development of improvements In an attempt to counteract this trend, we have greatly increased the amount of background material at the beginning of each chapter of the manual and we have provided full references Users of the manual who read this material should have no problem understanding either the general design or the specific details of the experimental protocols We also strongly recommend reading the relevant protocols in their entirety before commencing work This allows reagents to be prepared ahead of time and permits the investigator to carry out the protocol efficiently.

This manual could not have been produced without the help and encouragement of a large number of people We are extremely grateful to colleagues from the University of Texas Southwestern Medical Center at Dallas, from Genetics Institute, from Harvard University, and from many other institutions who have read individual chapters, have submitted protocols, and have made many invaluable suggestions that have immeasurably improved the manual. We have listed all these individuals with their affiliations in a special section at the end of this preface.

We owe a special debt of thanks to Rick Myers and Alison Cowie of the University of California at San Francisco, who read all of the chapters in draft, eliminated many embarrassing errors, and made many valuable suggestions for improvements; to Winship Herr of Cold Spring Harbor Laboratory and Mary-Jane Gething of Southwestern Medical Center, who drafted the chapters on DNA sequencing and expression of cloned genes in mammalian cells, respectively; and to Judy Campbell, who contributed significantly to the preparation of the chapter on enzymes We are also grateful to Mike Ockler, who provided all of the conceptual illustrations in this edition, and to Carolyn Doyle, who assembled the index

Nina Irwin has played a special role in the writing of this manual Among her many contributions are the *E coli* strain list and the maps of the many strains of bacteriophage λ, plasmids, cosmids, and bacteriophage M13 used in this manual These maps are based on many months of painstaking work to reconstruct and verify the genealogy of these host and vector strains In addition, Nina also drafted the chapter on expression of cloned genes in prokaryotic hosts, read and reviewed all of the chapters in manuscript and galleys, and searched the scientific literature with great intelligence and skill to validate many of the facts that appear

We also wish to thank Jim Watson for his continued interest in this manual. The support he has provided made this edition possible

Finally, we would like to express our deep appreciation of the work of our editor Nancy Ford For over two years, she has worked untiringly to bring order and sense to the entire project and to improve and clarify our writing Nancy and her assistant Michele Ferguson have both served as a source of encouragement, sympathy, and friendship We are greatly in their debt

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1

Plasmid Vectors

Bacterial plasmids are double-stranded closed circular DNA molecules that range in size from 1 kb to more than 200 kb They are found in a variety of bacterial species, where they behave as accessory genetic units that replicate and are inherited independently of the bacterial chromosome Nevertheless, they rely on enzymes and proteins encoded by the host for their replication and transcription. Frequently, plasmids contain genes coding for enzymes that under certain circumstances in nature are advantageous to the bacterial host Among the phenotypes conferred by plasmids are resistance to antibiotics; production of antibiotics, degradation of complex organic compounds; and production of colicins, enterotoxins, and restriction and modification enzymes.

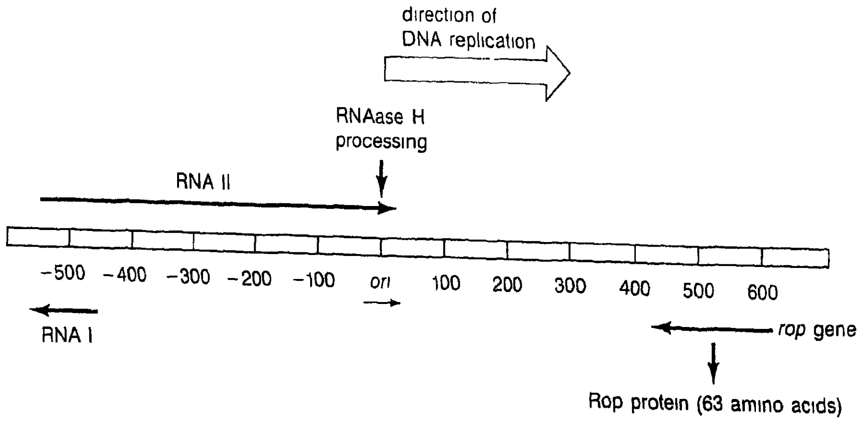


FIGURE 1.1

The figure shows the direction and approximate size of transcripts involved in the initiation of replication of plasmids carrying pMB1 (or ColE1) origins.

Essential Features of Plasmids

Replication and Incompatibility

Replication of plasmid DNA is carried out by subsets of enzymes used to duplicate the bacterial chromosome However, different plasmids use different subsets and replicate to different extents in their hosts. Some reach copy numbers as high as 700 per cell, whereas others are maintained at the minimal level of 1 plasmid molecule per host-cell chromosome. The control of plasmid copy number resides in a region of the plasmid DNA that includes the origin of DNA replication Usually, a plasmid will contain only one origin of replication together with its associated *cis*-acting control elements (the whole genetic unit being defined as a "replicon"). Very rarely, however, plasmids that have been generated by fusion will contain more than one replicon; in such cases, only one replicon is active

Most vectors in current use carry a replicon derived from the plasmid pMB1, which was originally isolated from a clinical specimen (Hershfield et al 1974) Under normal conditions of growth, a minimum of 15–20 copies of plasmids carrying this replicon (or its close relative the ColE1 replicon) (Bolivar et al 1977a,b) (see Table 1 1) are maintained in each bacterial cell (Covarrubias et al 1981) Such multicopy plasmids are said to replicate in a "relaxed" fashion The pMB1 (or ColE1) replicon does not require plasmid-encoded functions for replication (Tomizawa et al 1974); instead, it relies entirely on long-lived enzymes supplied by the host (DNA polymerases I and III, DNA-dependent RNA polymerase, and the products of the host genes *dnaB, dnaC, dnaD,* and *dnaZ*) (for review, see Scott 1984) It can therefore function in the absence of ongoing protein synthesis (for review, see Staudenbauer 1978) Thus, in the presence of antibiotics such as chloramphenicol or spectinomycin, which inhibit protein synthesis and prevent replication of the bacterial chromosome, plasmids carrying the pMB1 (or ColE1) replicon will continue to replicate until two or three thousand copies have accumulated in the cell (Clewell 1972)

Replication occurs unidirectionally from a specific origin and is primed by an RNA primer whose promoter lies about 550 bases upstream of the origin of replication (Figure 1 1) Persistent hybrids formed between the template strand of DNA and the nascent RNA serve as substrates for the enzyme RNAase H, which cleaves the preprimer to generate the primer for DNA synthesis (known as RNA II) (Itoh and Tomizawa 1980) The maturation of RNA II is controlled by another small, untranslated RNA molecule (RNA I), which is transcribed from the opposite strand of the same region of DNA that codes for RNA II RNA I binds to RNA II and prevents its folding into a cloverleaf structure that is necessary for the formation of stable DNA:RNA hybrids between RNA II and the plasmid DNA (see Tomizawa 1984; Dooley and Polisky 1987). This binding between RNAs I and II is enhanced by a small protein of 63 amino acids (the Rop protein), which is encoded by a gene located 400 nucleotides downstream from the origin of replication (Cesareni et al. 1982; Tomizawa and Som 1984). Consequently, the Rop protein reinforces the negative regulation of replication by RNA I (for review, see Cesareni and Banner 1985).

The copy number of plasmids carrying the pMB1 (or ColE1) replicon can therefore be increased by mutations that weaken the interaction between RNAs I and II. These mutations map within the *rop* gene or within the region upstream of the origin that codes for RNAs I and II (for review, see Scott 1984). For example, pUC plasmids have a high copy number because they carry a mutation (G→A) one nucleotide upstream of the normal site of initiation of RNA I (Minton et al. 1988). This results in RNA I transcripts that are initiated three nucleotides further downstream than usual. The integrity of the 5′ single-stranded domain of RNA I is crucial for the interaction of RNA I with RNA II (Dooley and Polisky 1987). It therefore seems likely that the increased copy number of pUC plasmids is caused by inefficient binding of truncated RNA I to RNA II.

In addition to controlling copy number, the regions of the plasmid encoding RNA I, RNA II, and the Rop protein also determine whether two different plasmids will coexist in the same bacterial cell (for review, see Davison 1984). Plasmids that utilize the same replication system cannot coexist stably and are said to be incompatible (Datta 1979). When two such plasmids are introduced into the same cell, they compete with one another both during replication and during the subsequent step of partition into daughter cells. Because plasmid molecules are selected at random from the intracellular pool for replication, the copy numbers of two different plasmids in an individual cell will not always remain equal. Small differences, generated originally by stochastic processes, lead rapidly to more severe imbalances in the copy numbers of the two plasmids. In some cells, one plasmid dominates; in others, its incompatible partner prospers. Over the course of a few generations of bacterial growth, the minority plasmid is completely eliminated and the descendants of the original cell contain one plasmid or the other, but not both. By testing the ability of different plasmids to coexist in the same cell, it is possible to assign them to incompatibility groups. Plasmids carrying the same replicon belong to the same incompatibility group; plasmids carrying replicons whose components are not interchangeable belong to different groups. Over 30 such groups have now been recognized (Datta 1979).

The replicons carried by plasmid vectors in current use are shown in Table 1.1. Plasmids carrying the ColE1 and pMB1 replicons are incompatible with one another, but they are fully compatible with those carrying pSC101 and p15A replicons (Chang and Cohen 1978; Selzer et al. 1983). The pSC101 replicon, which has not been analyzed in great detail, may resemble the replicon of plasmid R6-5, from which it is partially derived (Cohen and Chang

TABLE 1.1 Replicons Carried by Currently Used Plasmid Vectors

Plasmid	Replicon	Copy number
pBR322 and its derivatives	pMB1	15–20
pUC vectors	pMB1	500–700
pACYC and its derivatives	p15A	10–12
pSC101 and its derivatives	pSC101	~5
ColE1	ColE1	15–20

1977). The R6-5 replicon differs from the others shown in Table 1.1 in that it codes for an essential *cis*-acting protein (Linder et al. 1985; Womble et al. 1985). This protein acts positively on the origin of replication and regulates negatively the transcription of its own gene (the *repA* gene). Plasmids like pSC101 are therefore under stringent replicative control and are present at only 5 copies per cell or less. Because stringently controlled replication requires ongoing expression of a plasmid-encoded protein, the copy number of these plasmids cannot be increased by inhibitors of protein synthesis such as chloramphenicol. Most cloning tasks are therefore carried out in relaxed vectors because they give a greater yield of DNA per unit volume of culture and, in many cases, an improved yield of protein products synthesized from cloned genes. However, under some circumstances, the presence of many copies of the cloned gene and/or its product may be deleterious. The use of stringently controlled plasmids may then permit the isolation of functional genes that are lethal when expressed in high amounts from a multicopy plasmid. Examples of such genes include *polA* (Murray and Kelley 1979), *dnaA* (Hansen and von Meyenburg 1979), *ompA* (Beck and Bremer 1980), and *galK* (Davison et al. 1984). For descriptions of low-copy-number plasmid vectors, see Kahn et al. (1979) and Stoker et al. (1982).

Mobilization

Under natural conditions, many plasmids are transmitted to new hosts by a process known as bacterial conjugation (for review, see Willetts and Wilkins 1984). However, plasmid vectors in common use lack a gene (*mob*) that is required for mobilization and are incapable of directing their own conjugal transfer from one bacterium to another. Nevertheless, some of the older plasmid vectors (e.g., pBR322) can be mobilized by a conjugative plasmid if a third plasmid (ColK) is present in the cell (Young and Poulis 1978). ColK is thought to code for a mobility protein that nicks pBR322 at a site (*nic*) close to the *cis*-acting element *bom*. Mobilization of the plasmid then occurs from this nicked site (nucleotide 2254 in the pBR322 sequence). Newer plasmid vectors (e.g., the pUC vectors) lack the *nic/bom* site and therefore cannot be mobilized (Twigg and Sherratt 1980, Covarrubias et al. 1981).

Selectable Markers

In the laboratory, plasmid DNA can be introduced into bacteria by the artificial process of transformation. In this process, bacteria are treated with mixtures of divalent cations to make them temporarily permeable to small DNA molecules. Even under the best conditions, plasmids become stably established in only a small minority of the bacterial population. To identify these transformants, selectable markers encoded by the plasmid are used. These markers confer a new phenotype, which allows bacteria that have been successfully transformed to be selected with ease.

The most commonly used selectable markers are genes that confer resistance to antibiotics such as ampicillin, tetracycline, chloramphenicol, and kanamycin (neomycin). Each of these antibiotics operates through a different mechanism (for review, see Davies and Smith 1978):

- *Tetracycline* binds to a protein of the 30S subunit of the ribosome and inhibits ribosomal translocation The constitutively expressed tetracycline resistance (*tet*^{r}) gene carried on plasmid pBR322 encodes a 399-amino-acid, membrane-associated protein (Backman and Boyer 1983) that prevents the antibiotic from entering the cell (Franklin 1967)

- *Ampicillin* binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the cell wall (for review, see Waxman and Strominger 1983) The ampicillin resistance (*amp*^{r}) gene carried on the plasmid codes for an enzyme that is secreted into the periplasmic space of the bacterium, where it catalyzes hydrolysis of the β-lactam ring, with concomitant detoxification of the drug (Sykes and Mathew 1976)

- *Chloramphenicol* binds to the ribosomal 50S subunit and inhibits protein synthesis The chloramphenicol resistance (*Cm*^{r} or *cat*) gene used in current plasmid vectors was originally isolated from a transducing P1 bacteriophage (Kondo and Mitsuhashi 1964) that was later shown to carry the transposon Tn*9* The *cat* gene codes for a tetrameric, cytosolic protein (M_r of each subunit = 23,000) that, in the presence of acetyl coenzyme A, catalyzes the formation of hydroxyl acetoxy derivatives of chloramphenicol that are unable to bind to ribosomes (Shaw et al 1979) The expression of chloramphenicol acetyltransferase is catabolite-sensitive and is increased five- to tenfold when bacteria are grown on carbon sources other than glucose Binding of DNA-dependent RNA polymerase to the promoter of the *cat* gene in vitro is increased markedly by the presence of the cAMP/catabolite gene activator protein (Le Grice and Matzura 1981)

- *Kanamycin and neomycin* are deoxystreptamine aminoglycosides that bind to ribosomal components and inhibit protein synthesis Both antibiotics are inactivated by an aminoglycoside phosphotransferase (APH[3']-II) with a molecular weight of 25,000 that appears to be located in the periplasmic space Phosphorylation of these antibiotics is believed to interfere with their active transport into the cell (for review, see Davies and Smith 1978)

Virtually all plasmid vectors in common use carry one or more of the antibiotic resistance genes described above However, other selectable markers are occasionally used for more specialized purposes Perhaps the most elegant of these is *supF*, the gene that codes for the bacterial suppressor tRNA (Seed 1983) This small (203-bp) gene is used in the plasmid πVX (Seed 1983, see Maniatis et al 1982 for map) and its updated version πAN13 (Lutz et al 1987) to suppress amber mutations in *tet*^{r} and *amp*^{r} genes carried on a second, compatible plasmid. This suppression confers ampicillin and tetracycline resistance upon cells containing both plasmids

Plasmid Vectors

DEVELOPMENT OF PLASMID CLONING VECTORS

The development of plasmid vectors has proceeded in three phases:

1 Selectable markers were introduced into plasmids carrying the pMB1 (or ColE1) replicon The first plasmids used as cloning vectors—pSC101 (Cohen et al 1973), ColE1 (Hershfield et al. 1974), and pCR1 (Covey et al. 1976)—were limited in their versatility: Either they replicated poorly or they carried unsuitable selectable markers. None of them contained more than two restriction sites that could be used for cloning. The first plasmid to combine all of the then-available desirable features was pBR313 (Bolivar et al 1977a,b) It replicated in a relaxed fashion, contained two selectable markers (*tet*^{r} and *amp*^{r}), and carried a number of useful restriction sites However, pBR313 was unnecessarily large, more than half of its DNA was not essential for its role as a vector. The first phase of plasmid vector development ended with the construction of pBR322 (Bolivar et al 1977b), a plasmid of 4 36 kb from which most of these unnecessary sequences had been eliminated pBR322 rapidly became the most widely used cloning vehicle, and many of the plasmid vectors in use today are its direct descendants (for review, see Balbás et al 1986)

2. The trend for the next few years was to streamline vectors to reduce their size to the minimum while expanding their capacity to accept fragments of foreign DNA generated by cleavage with a wide range of restriction enzymes Smaller plasmid vectors are preferred for many reasons. First, the efficiency of transformation is inversely related to the size of the plasmid This becomes a limiting factor when the plasmid exceeds 15 kb in size Smaller plasmids can therefore accommodate larger segments of foreign DNA before the efficiency of transformation begins to deteriorate. Second, larger plasmids are more difficult to characterize by restriction mapping Third, because larger plasmids replicate to lower copy numbers, the yield of foreign DNA is reduced and the strength of signals obtained when colonies are screened by hybridization is reduced Thus, in the late 1970s and early 1980s, a number of derivatives of pBR322 were constructed that lacked ancillary sequences involved in the control of copy number and mobilization. The best known of these are pAT153 (Twigg and Sherratt 1980), pXf3 (Hanahan 1983), and pBR327 (Soberón et al. 1980), all of which remain in common use (See Maniatis et al [1982] for maps of pAT153 and pXf3)

   At the same time, the number of useful restriction enzyme cleavage sites within plasmid vectors was expanded and their distribution was rationalized. Almost all vectors now contain a closely arranged series of synthetic cloning sites, termed "polylinkers," "restriction site banks," or "polycloning sites," that consist of sequences recognized by restriction enzymes commonly used in cloning experiments. In most cases, these restriction sites are unique—i.e., they are not found elsewhere in the plasmid vector. For example, the polycloning site from the vector pUC19 consists of a tandem array of cleavage sites for 13 restriction enzymes *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Acc*I, *Hin*cII, *Xba*I, *Bam*HI, *Sma*I, *Xma*I, *Kpn*I, *Sac*I, and *Eco*RI (see page 1 13).

Such an array of recognition sequences provides a vast variety of targets that can be used singly or in combination to clone DNA fragments generated by cleavage with any of a very large number of restriction enzymes. Furthermore, fragments inserted at one restriction site can often be excised by cleavage of the recombinant plasmid with restriction enzymes that cleave at flanking sites Insertion of a segment of DNA into a polycloning site is therefore equivalent to adding synthetic linkers to its termini The availability of these flanking sites simplifies the task of mapping the segment of foreign DNA.

A disadvantage of drawing together all potential cloning sites into one location in a plasmid is the inability to use inactivation of a selectable marker to screen for recombinants In this method, which can be used with plasmids that carry two or more different selectable markers, a restriction site located within one of the markers is used to clone a segment of foreign DNA In most cases, insertion of the foreign sequences inactivates the marker Transformants containing recombinant plasmids can therefore be distinguished from those carrying the parental vector by virtue of their inability to grow under one of the two sets of selective conditions (see Figure 1 12, page 1 88) The inability to use insertional inactivation of antibiotic resistance markers with modern vectors has been largely overcome by incorporating a cluster of cloning sites into a gene that codes for a biologically active fragment of an enzyme such as β-galactosidase. Insertion of a segment of foreign DNA almost always leads to the production of an inactive fragment Because recombinant clones lose their ability to hydrolyze a chromogenic substrate, they can therefore be distinguished from parental clones by a simple, nondestructive histochemical test (see below).

3 The latest phase of construction of plasmid vectors has involved the incorporation of ancillary sequences that are used for a variety of purposes, including visual identification of recombinant clones by histochemical tests, generation of single-stranded DNA templates for DNA sequencing, transcription of foreign DNA sequences in vitro, direct selection of recombinant clones, and expression of large amounts of foreign proteins These specialized functions are discussed only briefly here, but many of them are described in detail in later chapters

Plasmid Vectors That Permit Histochemical Identification of Recombinant Clones

The best-known vectors of this class are the pUC plasmids developed by Messing and his colleagues (Vieira and Messing 1982, Norrander et al. 1983; Yanisch-Perron et al. 1985). These vectors carry a segment of DNA derived from the *lac* operon of *Escherichia coli* that codes for ~~the~~ amino-terminal fragment of β-galactosidase. This fragment, whose synthesis can be induced by isopropylthio-β-D-galactoside (IPTG), is capable of intra-allelic (α) complementation with a defective form of β-galactosidase encoded by the host. Bacteria exposed to the gratuitous inducer IPTG synthesize both fragments of the enzyme and form blue colonies when plated on media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Horwitz et al. 1964). Insertion of foreign DNA into the polycloning site of the plasmid inactivates the amino-terminal fragment of β-galactosidase and abolishes α-complementation. Bacteria carrying recombinant plasmids therefore give rise to white colonies. (See page 1.85 for a discussion of screening by α-complementation.)

Plasmid Vectors Carrying Origins of Replication Derived from Single-stranded Bacteriophages

Several plasmid vectors have been constructed that carry the origin of replication from the genome of a single-stranded filamentous bacteriophage such as M13 (see Chapter 4). This allows copies of one of the two strands of the plasmid DNA to be produced in quantities sufficient for sequencing (see Chapter 13) or for preparation of radiolabeled single-stranded probes (see Chapter 10).

Plasmid Vectors Carrying Bacteriophage Promoters

Many plasmid vectors carry promoters derived from bacteriophages T3, T7, and/or SP6 adjacent to the polycloning site. Foreign DNAs inserted at restriction sites can therefore be transcribed in vitro when the linearized recombinant plasmid DNA is incubated with the appropriate DNA-dependent RNA polymerase and ribonucleotide precursors (see Chapter 10). In some cases, a different promoter is present at each end of the polycloning site, which allows RNA to be transcribed from either end and either strand of the foreign DNA, depending on the type of RNA polymerase used in the transcription reaction. The RNAs generated in this way can be used as hybridization probes or can be translated in cell-free protein-synthesizing systems.

Plasmid Vectors That Allow Direct Selection of Recombinant Clones

A number of selective systems have been designed to suppress the growth of bacteria carrying nonrecombinant plasmids. Typically, the plasmids used in these systems express a gene product that is lethal for certain bacterial hosts; insertion of a segment of foreign DNA inactivates the gene. For example, Maloy and Nunn (1981) and Craine (1982) have described conditions under which transformed bacteria carrying plasmid vectors coding for *tet*^{r} will die, whereas recombinant plasmids carrying a segment of foreign DNA within the *tet*^{r} gene will grow. Other selective systems depend on insertional inactivation of genes such as those encoding bacteriophage λ repressor (Roberts et al. 1980), *Eco*RI methylase (Cheng and Modrich 1983), galactokinase (Ahmed 1984), and colicin E3 (Vernet et al. 1985). In our experience, the disadvantages of these systems generally outweigh the advantages. The number of potential cloning sites is usually limited, the efficiency of the selection is variable, special host cells are often required, and the plasmids are frequently devoid of desirable features (bacteriophage promoters, bacteriophage M13 origin of DNA replication, etc.) For lists of the available selective systems and brief descriptions of their properties, see Burns and Beacham (1984) and Balbás et al (1986).

Plasmid Expression Vectors

A large number of plasmid vectors have been constructed that contain powerful promoters that generate large amounts of mRNA complementary to cloned sequences of foreign DNA In some cases, these vectors are designed to express foreign proteins that are not linked to any prokaryotic sequences, in others, they generate fusion proteins encoded partly by the vector and partly by an open reading frame in the cloned segment of foreign DNA These vectors and their uses are described in detail in Chapter 17

COMMONLY USED PLASMID VECTORS

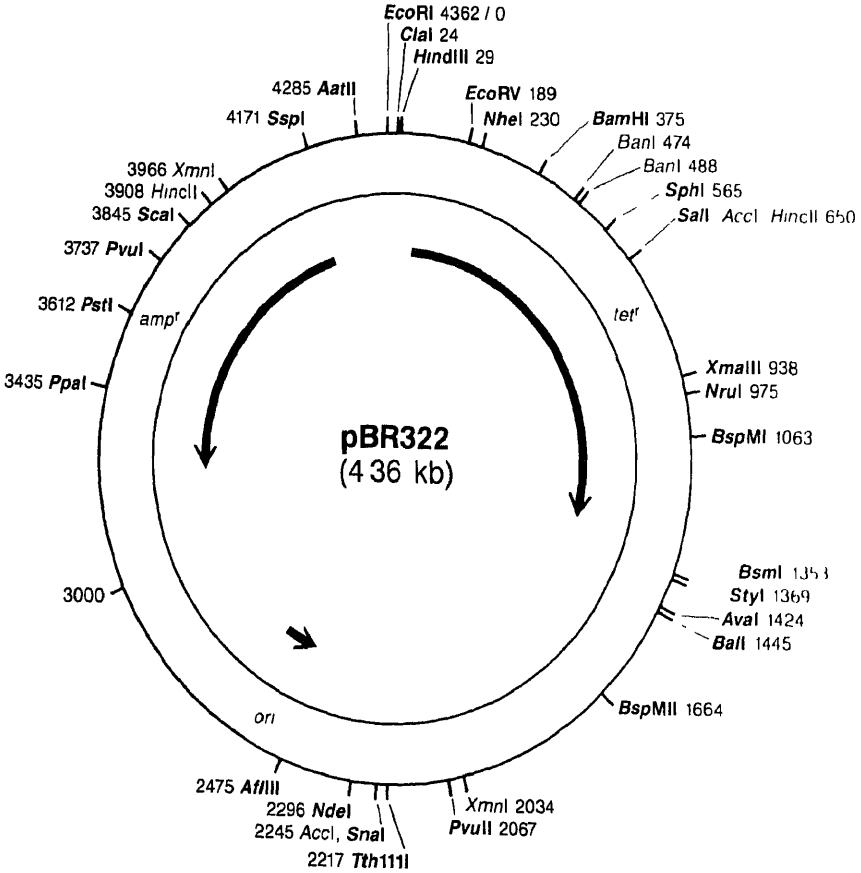
Maps of widely used plasmid vectors that contain many of the useful features described earlier in this chapter are presented on the following pages. Each of these vectors has been completely sequenced. The maps have been prepared based on the information contained in the references given at the end of the description of each vector Plasmid vectors used for expression of foreign proteins in *E coli* are discussed in Chapter 17. Vectors used to generate single-stranded DNA for sequencing are described in detail in Chapter 4

pBR322

pBR322 is presented because it served for many years as the workhorse plasmid vector It has, however, been largely superseded by improved, more efficient vectors

Note pBR322 and some of its derivatives form multimers in $recA^+$ hosts (Bedbrook et al 1979) and are readily lost from cells during sustained growth in minimal media (Jones et al 1980, Noack et al 1981, Skogman et al 1983) This occurs because pBR322 does not carry a locus (*cer*) that ensures equal partitioning of the plasmid into daughter cells (Summers and Sherratt 1984) Whenever possible, bacteria containing pBR322-based vectors or recombinants should therefore be grown in selective conditions Derivatives of pBR322 containing the *par* region of pSC101 are, however, available (Zurita et al 1984) *par,* like *cer,* confers a stable pattern of plasmid inheritance (Skogman et al 1983)

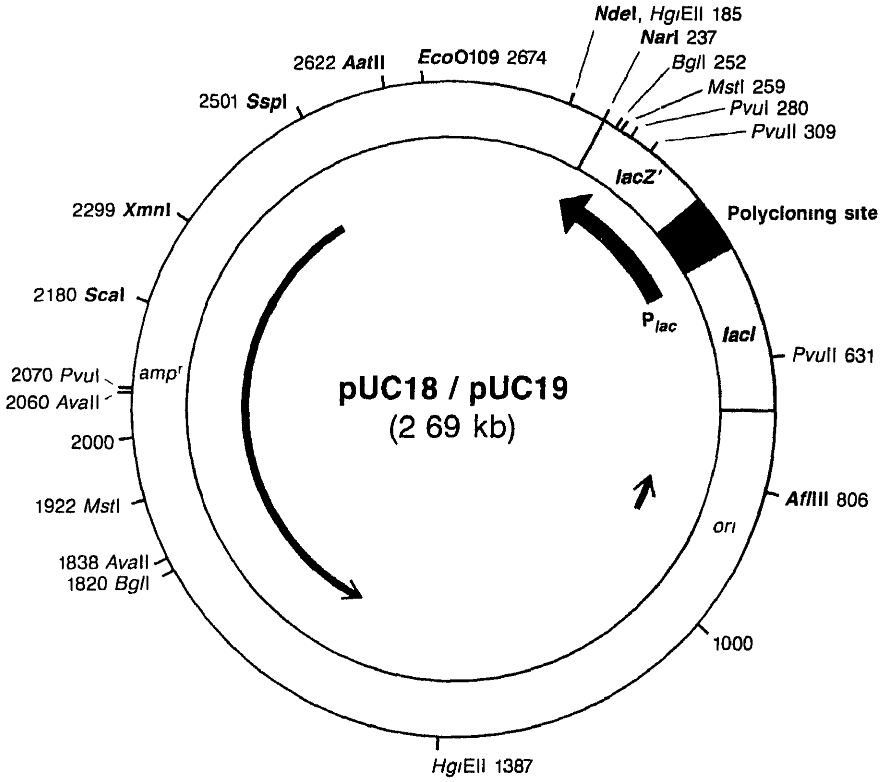
References Bolivar et al (1977b) and Sutcliffe (1979)



pUC18, pUC19

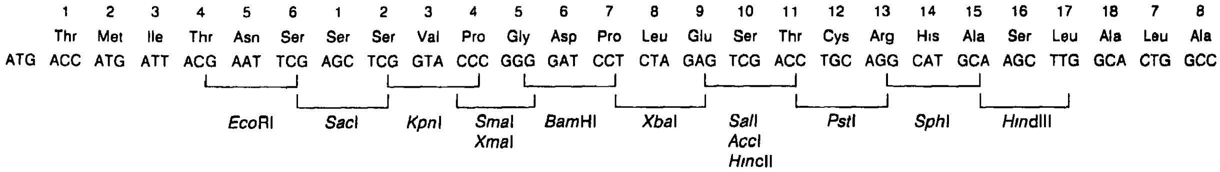
These vectors are identical except that they contain polycloning sites arranged in opposite orientations pUC plasmids lack the *rop* gene (see page 1 3), which normally is located close to the origin of DNA replication and is involved in the control of copy number As a result, these plasmids replicate to a much higher copy number than do other plasmids that carry a pMB1 (or ColE1) origin. pUC vectors express the amino-terminal fragment of the *lacZ* gene product (β-galactosidase) and display α-complementation in appropriate hosts (see pages 1 8–1.9) Recombinants can therefore be identified by histochemical screening

References Messing (1983), Norrander et al (1983), and Yanisch-Perron et al (1985)



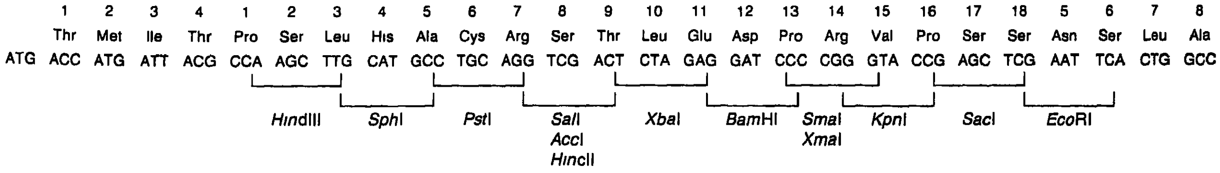
Polycloning Sites

pUC18



	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC

pUC19



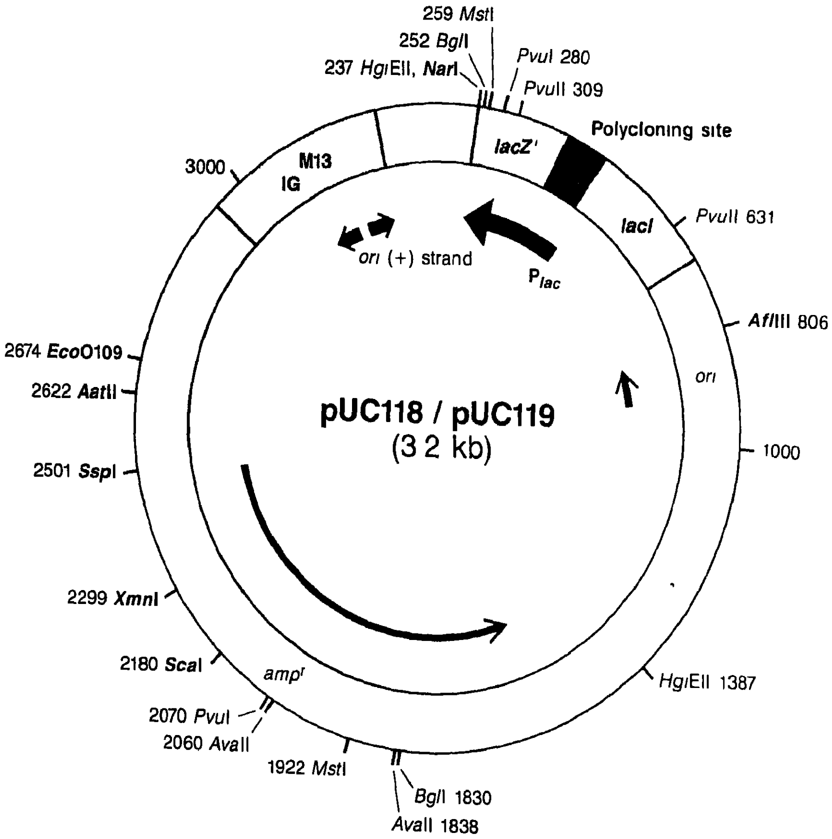
	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC

In pUC18, the *Eco*RI site lies immediately downstream from P_{lac}
In pUC19, the *Hin*dIII site lies immediately downstream from P_{lac}

pUC118, pUC119

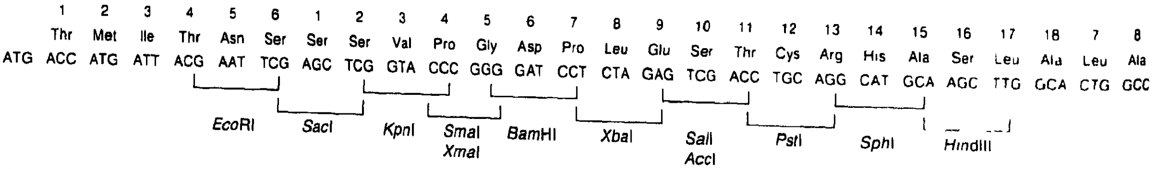
Derived from pUC18 and pUC19, pUC118 and pUC119 carry the sequences required in *cis* for initiation and termination of bacteriophage M13 DNA synthesis and for packaging of DNA into bacteriophage particles These vectors can accept segments of foreign DNA and can be propagated as plasmids in the conventional way When cells harboring these plasmids are infected with a suitable filamentous bacteriophage, copies of one strand of the plasmid DNA are synthesized and packaged into progeny bacteriophage particles (see Chapter 4) Single-stranded DNA can be isolated from the bacteriophage particles and used as a template to determine the nucleotide sequence of the foreign DNA, for oligonucleotide-directed mutagenesis, and for synthesis of strand-specific probes

References Messing (1983), Norrander et al (1983), Yanisch-Perron et al (1985), and Vieira and Messing (1987)



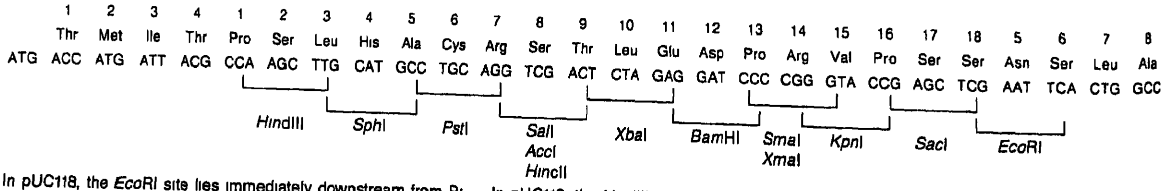
Polycloning Sites

pUC118



	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC

pUC119



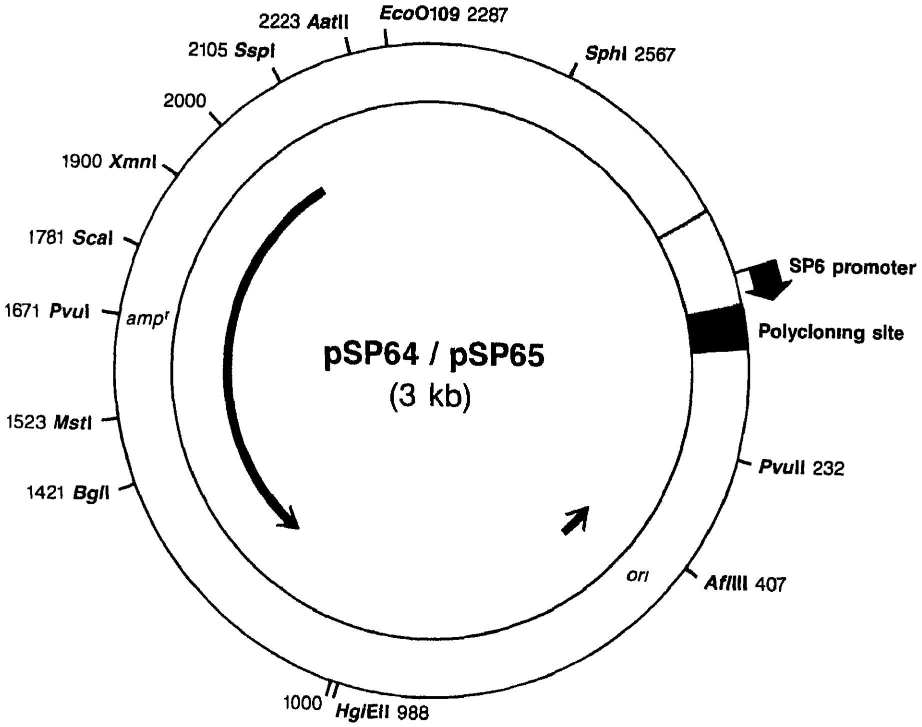
	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC

In pUC118, the *Eco*RI site lies immediately downstream from P*lac* In pUC119, the *Hin*dIII site lies immediately downstream from P*lac*

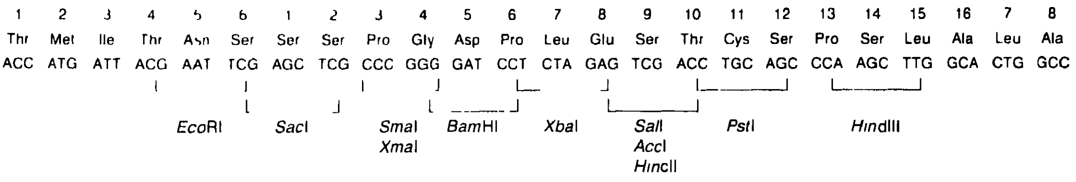
pSP64, pSP65, pGEM-3, pGEM-3Z, pGEM-3Zf(−), pGEM-4, pGEM-4Z

These vectors are derivatives of pUC plasmids that carry promoters for bacteriophage-encoded DNA-dependent RNA polymerase transcription units Derivatives of pSP64 and pSP65 are available (pSP64CS and pSP65CS; Eckert 1987) that have been designed to facilitate sequencing of DNA by the Maxam-Gilbert method (see Chapter 13)

References pSP64 and pSP65 Melton et al (1984), pGEM series: Promega Catalogue and Reference Guide—Biological Research Products (1987/1988)



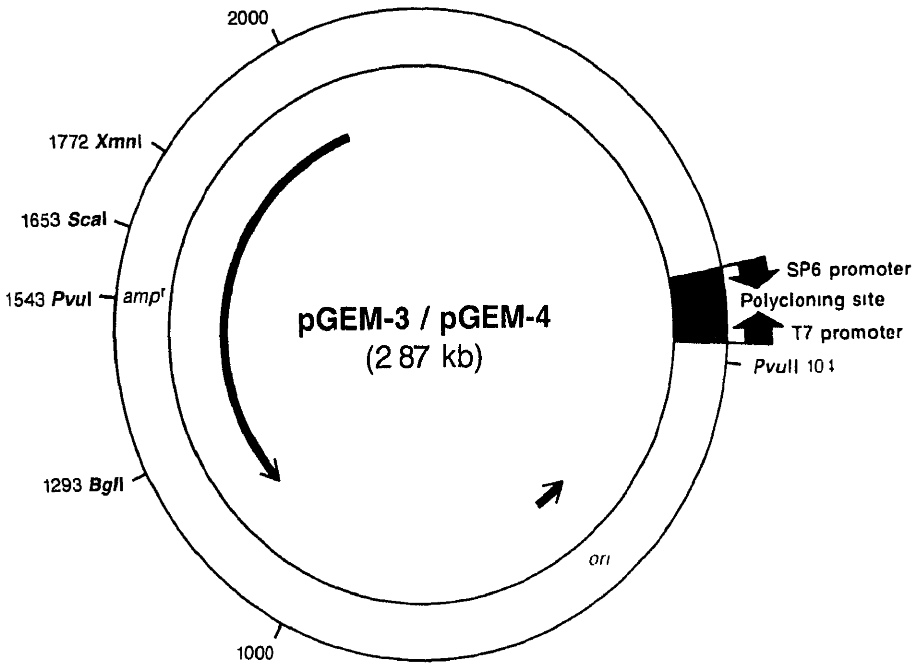
Polycloning Site

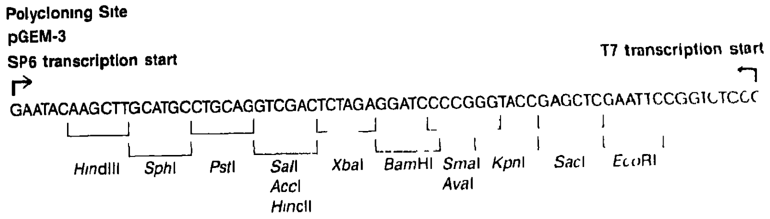


1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	7	8
Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Ser	Pro	Ser	Leu	Ala	Leu	Ala
ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGC	CCA	AGC	TTG	GCA	CTG	GCC

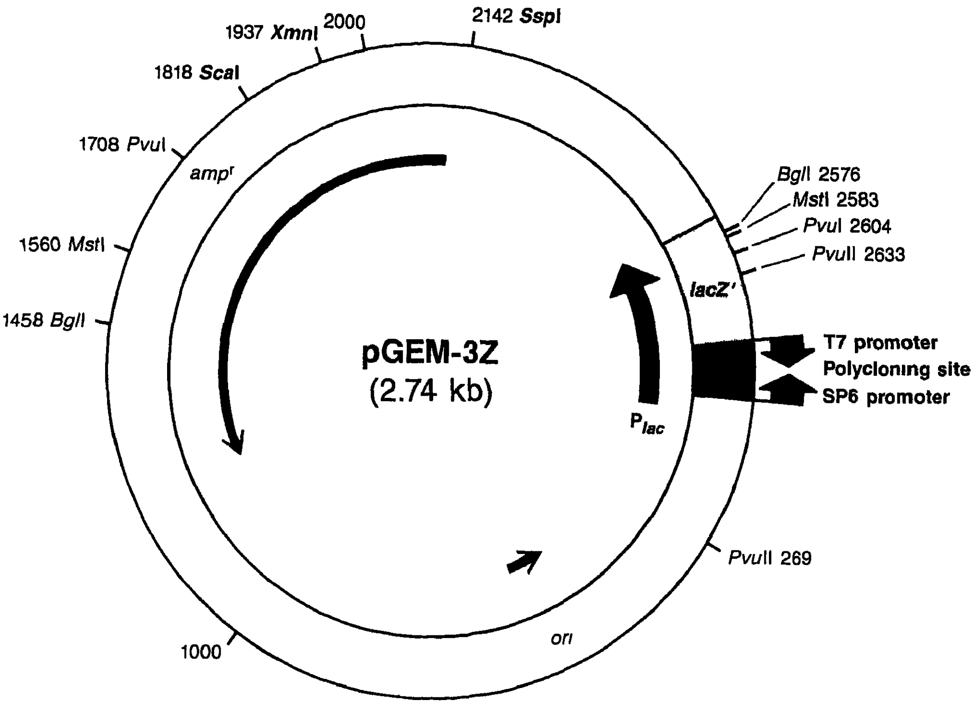
In pSP65 the *Eco*RI site lies immediately downstream from the SP6 promoter

In pSP64 the polycloning site is in the opposite orientation and the *Hind*III site lies immediately downstream from the SP6 promoter





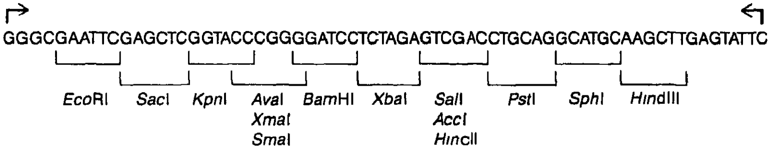
In pGEM 4 the polycloning site is in the opposite orientation



Polycloning Sites

pGEM-3Z

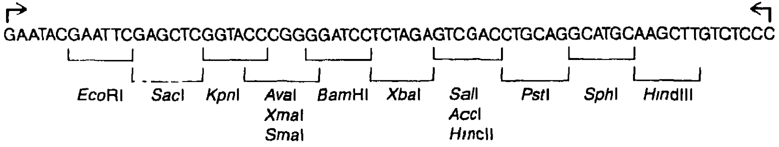
T7 transcription start **SP6 transcription start**



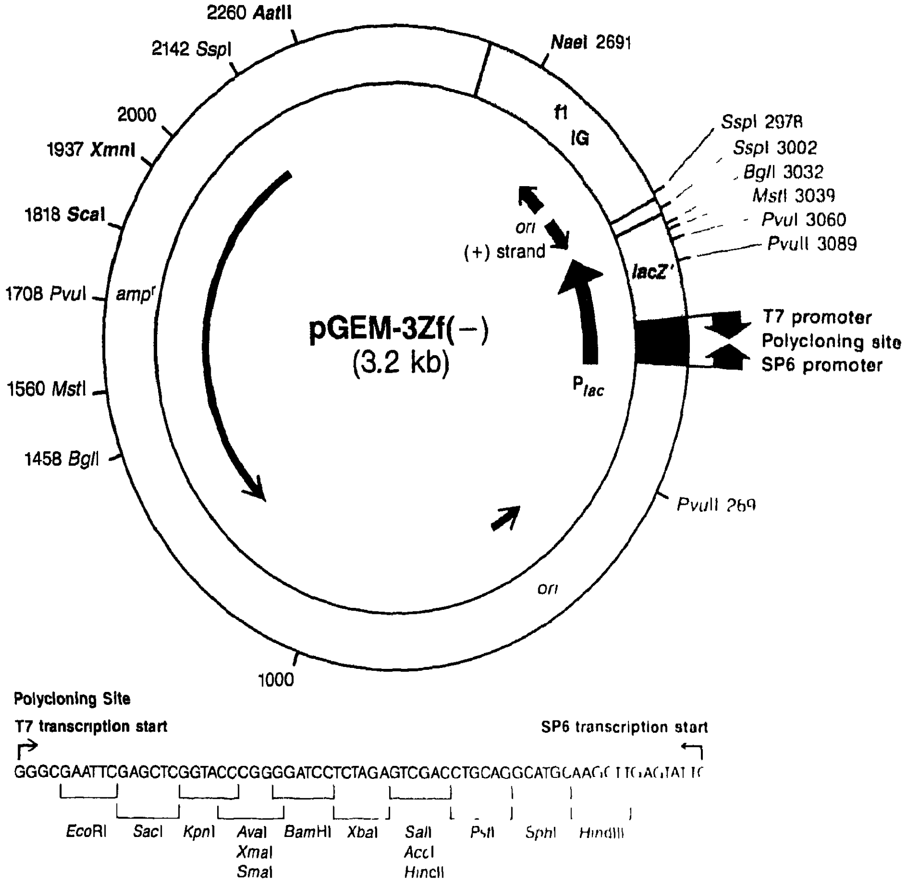
GGGCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATTC

pGEM-4Z

SP6 transcription start **T7 transcription start**



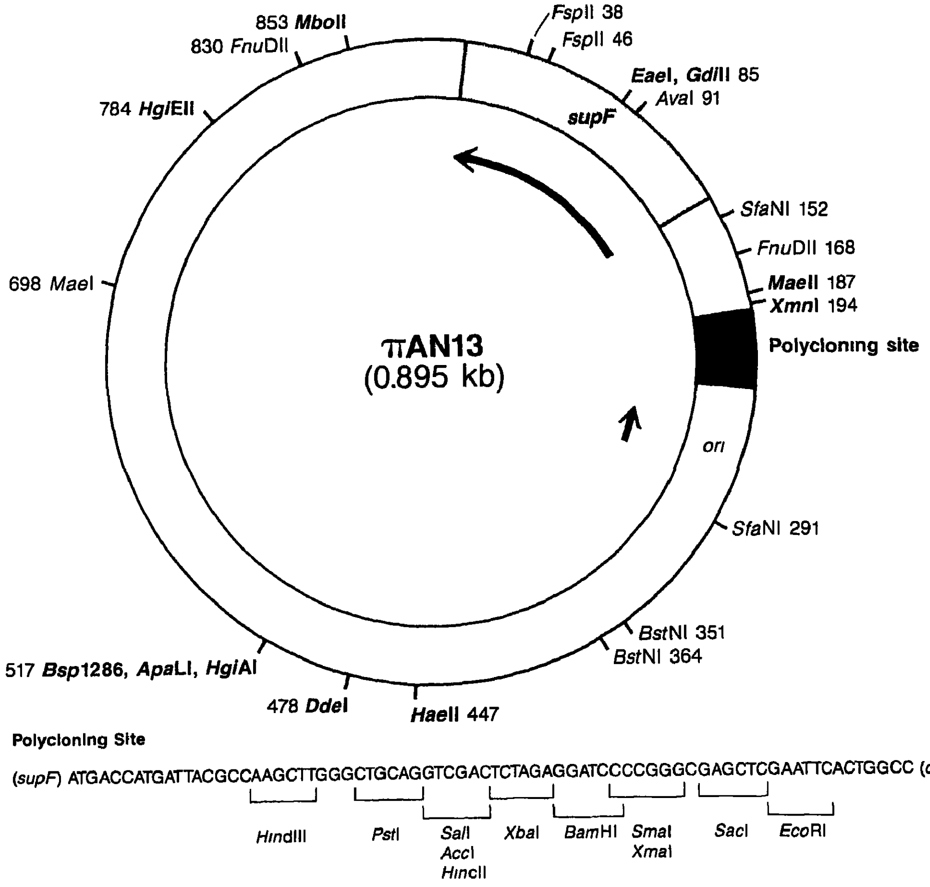
GAATACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGTCTCCC


2260 AatII
2142 SspI
2000
1937 XmnI
1818 ScaI
1708 PvuI
1560 MstI
1458 BglI
1000
NaeI 2691
f1
IG
SspI 2978
SspI 3002
BglI 3032
MstI 3039
PvuI 3060
PvuII 3089
ori
(+) strand
lacZ'
T7 promoter
Polycloning site
SP6 promoter
pGEM-3Zf(−)
(3.2 kb)
P_{lac}
amp^r
PvuII 269
ori
Polycloning Site
T7 transcription start
SP6 transcription start
GGGCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATTC
EcoRI
SacI
KpnI
AvaI
XmaI
SmaI
BamHI
XbaI
SalI
AccI
HincII
PstI
SphI
HindIII

πAN13

πAN13 is a small plasmid vector derived from πAN7 (Lutz et al 1987) that is used for recombination screening (Seed 1983) "Probe" sequences are cloned into πAN13 and the recombinant plasmid is introduced into *E coli* Libraries constructed in bacteriophage λ vectors that carry several amber mutations in essential genes (e g , Syrinx 2A) are grown in bacteria containing the recombinant plasmid If the DNA sequence in the infecting bacteriophage is homologous to the probe sequence in πAN13, the plasmid can be inserted into the bacteriophage by in vivo homologous recombination In the resulting cointegrants, the *supF* in the πAN13 suppresses the amber mutations in the bacteriophage and thus allows plaque formation in nonsuppressing hosts. Members of multigene families, genes that encode cDNA, and loci from multiple genomic DNA libraries can be selected The original "recombination probe" plasmid πVX has a low copy number (see Maniatis et al 1982 for map of πVX) πAN13 was designed to increase copy number and thus increase recombination frequency πAN13 contains a pBR322 origin of replication, the pUC13 polycloning site, and a *supF* tRNA gene (Lutz et al 1987).

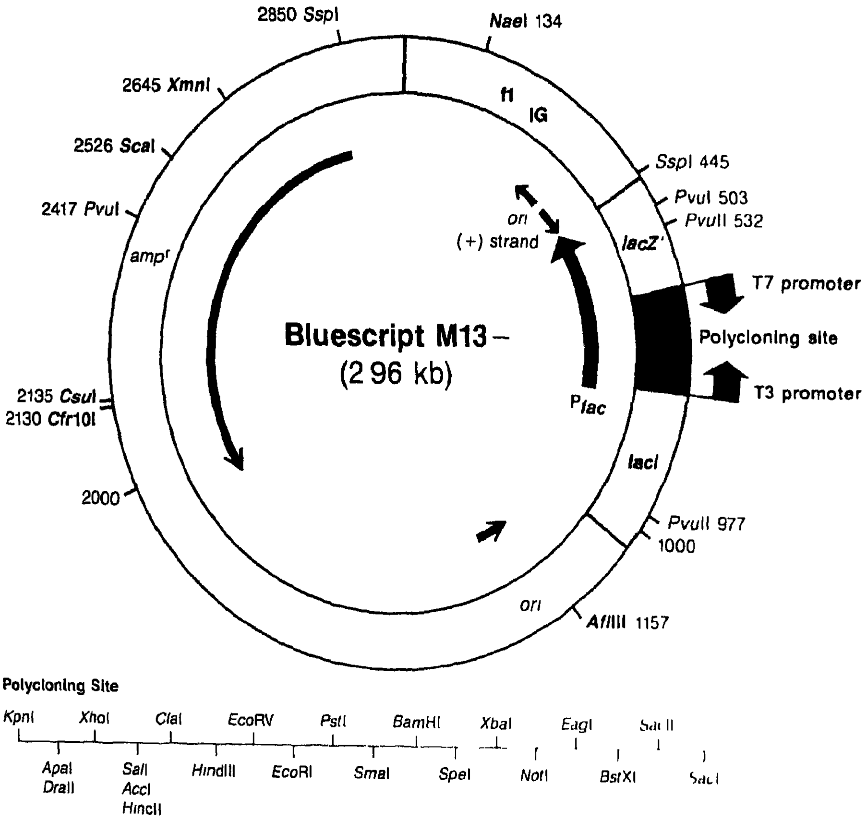
Reference Lutz et al (1987)



BLUESCRIPT M13 + , M13 −

Bluescript M13 + and M13 − are vectors that contain the bacteriophage M13 origin of DNA replication inserted in opposite orientations into a vector derived from a pUC plasmid that contains bacteriophage T3 and T7 promoters. These vectors can thus be used to generate single-stranded DNA in vivo, or RNA in vitro, that is complementary to either of the two strands of foreign DNA inserted into the polycloning site

References Bluescript M13 − Short et al (1988) and the product literature from Stratagene Cloning Systems



In Bluescript SK (M13−), the *SacI* site lies immediately downstream from the bacteriophage T3 promoter and the *KpnI* site lies immediately downstream from the bacteriophage T7 promoter In Bluescript KS (M13) the polycloning site is in the opposite orientation

Extraction and Purification of Plasmid DNA

Many methods have been developed to purify plasmid DNA from bacteria. These methods invariably involve three steps.

- Growth of the bacterial culture
- Harvesting and lysis of the bacteria
- Purification of plasmid DNA

Growth of the Bacterial Culture

Plasmids are almost always purified from cultures (grown in liquid medium containing the appropriate antibiotic) that have been inoculated with a single bacterial colony picked from an agar plate Many of the currently used plasmid vectors (e g , the pUC series) replicate to such a high copy number that they can be purified in large yield from cultures that have simply been grown to late log phase in standard LB medium. In these cases, it is not necessary to amplify the plasmid DNA selectively However, vectors of an earlier generation (e g , pBR322), which do not replicate so freely, need to be selectively amplified by incubating the partially grown bacterial culture in chloramphenicol for several hours. As discussed on page 1.3, chloramphenicol inhibits host protein synthesis and, as a result, prevents replication of the bacterial chromosome. However, replication of relaxed plasmids continues, and their copy number increases progressively for several hours The yield of plasmids such as pBR322 is thus considerably higher from chloramphenicol-treated cultures than from untreated cultures

For many years, it has been standard practice to add chloramphenicol in concentrations sufficient to achieve complete inhibition of protein synthesis (170 μg/ml) The yield of most plasmids isolated from cultures treated in this fashion is more than sufficient for almost any conceivable task in molecular cloning. However, in those cases where plasmids (or more commonly, cosmids) grow poorly because of their large size or because of the foreign sequences they carry, it may be worthwhile to explore alternative ways to grow and treat the bacterial culture For example, it has been reported (Tartof and Hobbs 1987) that the use of a rich medium (Terrific Broth; see Appendix A) results in a four- to sixfold increase in the yield of "difficult" plasmids. In addition, the concentration of chloramphenicol can affect the degree of amplification of plasmid DNAs. Improved yields of pBR322 and pBR327 have been obtained from bacterial cultures treated with concentrations of chloramphenicol (10–20 μg/ml) that do not completely suppress host protein synthesis (Frenkel and Bremer 1986). The reason for this result is not understood, but it could be explained if the replication of plasmids carrying the ColE1 origin required an unstable host factor that continues to be synthesized during partial inhibition of protein synthesis.

Harvesting and Lysis of the Bacteria

Bacteria are recovered by centrifugation and lysed by any one of a large number of methods, including treatment with nonionic or ionic detergents, organic solvents, alkali, or heat The choice among these methods is dictated by three factors. the size of the plasmid, the strain of *E coli*, and the technique that is to be used subsequently to purify the plasmid DNA. Although it is impractical to give precise conditions for each combination of plasmid and host, the following general guidelines can be used to select a method that will give satisfactory results

1 Large plasmids (> 15 kb in size), which are susceptible to damage, should be released from cells by gentle lysis (see page 1.36) Bacteria are suspended in an isosmotic solution of sucrose and then treated with lysozyme and EDTA to break down the cell wall and outer membrane The resulting spheroplasts are lysed by adding a detergent such as SDS This method minimizes the physical forces that are required to liberate the plasmid from the pressurized interior of the bacterium

2. More severe methods can be used to isolate smaller plasmids After addition of EDTA and, in some cases, lysozyme, the cells are exposed to detergent and lysed by boiling or treatment with alkali These treatments, which disrupt base pairing, cause the linear chromosomal DNA of the host to denature However, the strands of closed circular plasmid DNA are unable to separate from one another because they are topologically intertwined When conditions are returned to normal, the strands of the plasmid DNA rapidly fall into perfect register, and completely native superhelical molecules are re-formed

   Note Prolonged exposure of superhelical DNA to heat or alkali results in irreversible denaturation (Vinograd and Lebowitz 1966) The resulting cyclic coiled DNA cannot be cleaved with restriction enzymes, migrates through agarose gels at about twice the rate of superhelical DNA, and stains poorly with ethidium bromide Traces of this form of DNA can often be seen in plasmids prepared by alkaline or thermal lysis of bacteria

3 Some strains of *E coli* (e g , some variants and derivatives of HB101) release relatively large amounts of carbohydrate when they are lysed by detergent and heat This can be a nuisance when the plasmid DNA is subsequently purified by equilibrium centrifugation in CsCl–ethidium bromide gradients. The carbohydrate forms a dense, fuzzy band close to the place in the gradient occupied by the supercoiled plasmid DNA It is therefore difficult to avoid contaminating the plasmid DNA with carbohydrate, which inhibits the activity of many restriction enzymes Boiling should therefore not be used when making large-scale preparations of plasmids from strains of *E. coli* such as HB101 and TG1

4. The boiling method is not recommended when making small-scale preparations of plasmid DNA from strains of *E coli* that express endonuclease A (*endA*$^+$ strains; e.g , HB101). Because endonuclease A is not completely inactivated by the boiling procedure, the plasmid DNA is degraded during subsequent incubation in the presence of Mg^{++} (e.g., during digestion with restriction enzymes). This problem can be avoided by including an extra

step—extraction with phenol:chloroform—in the protocol given on page 1 29

5. Virtually all of the procedures given in this section can be easily adapted to accommodate bacterial cultures that range in size from 1 ml to 1 liter. All of them have been used successfully to isolate plasmid DNA simultaneously from many small cultures ("minipreps"), as well as from large-scale cultures ("maxipreps") and cultures of intermediate size ("midipreps"). With the one exception referred to in number 3 above, any method can therefore be used with any strain of *E. coli* grown in cultures of any size. It is curious, however, that many laboratories use one method to process minipreps and another method to isolate plasmid DNA from large-scale cultures. In some laboratories, the alkali method is used for minipreps and boiling is used for maxipreps, whereas in others, the situation is reversed

6 As discussed on pages 1 3–1 4, the copy numbers of the current generation of plasmids are now so high that selective amplification in the presence of chloramphenicol is unnecessary to achieve high yields. However, some workers continue to use chloramphenicol, not to increase the yield of plasmid DNA but to reduce the bulk of bacterial cells in maxipreps. Handling large quantities of highly viscous lysates of concentrated suspensions of bacteria is a frustrating and messy business that can be avoided if chloramphenicol is added to the culture at mid-log phase. Approximately equivalent yields of plasmid DNA are obtained from smaller numbers of cells that have been exposed to chloramphenicol and from larger numbers of cells that have not.

Purification of Plasmid DNA

All of the methods in common use exploit the relatively small size and covalently closed circular nature of plasmid DNAs For example, separation of plasmid and chromosomal DNAs by equilibrium centrifugation in CsCl–ethidium bromide gradients depends on differences between the amounts of ethidium bromide that can be bound to linear and closed circular DNA molecules. Ethidium bromide binds to DNA by intercalating between the bases, causing the double helix to unwind This leads to an increase in the length of linear DNA molecules and to the introduction of compensatory superhelical turns in closed circular plasmid DNAs Eventually, the density of these superhelical turns becomes so great that the intercalation of additional molecules of ethidium bromide is prevented Linear molecules, which are not constrained in this way, continue to bind more dye until saturation is reached (~1 ethidium bromide molecule for every 2 base pairs) (Cantor and Schimmel 1980) Because of this differential binding of dye, the buoyant densities of the linear and closed circular DNA molecules are different in CsCl gradients containing saturating amounts of ethidium bromide

For many years, equilibrium centrifugation in CsCl–ethidium bromide gradients has been the method of choice to prepare large amounts of plasmid DNA. However, this process is expensive and time-consuming, and many alternative methods have been developed, the majority of which involve the use of ion-exchange or gel-filtration chromatography or differential precipitation to separate plasmid and host DNAs. Although most of these methods have fallen by the wayside, the best of them—differential precipitation with polyethylene glycol—has recently been improved (R Treisman, pers comm) to the point where it yields plasmid DNA of extremely high purity Differential precipitation with polyethylene glycol differs from equilibrium centrifugation in CsCl–ethidium bromide gradients in one respect It does not efficiently separate nicked circular molecules from the closed circular form of plasmid DNA Equilibrium centrifugation is therefore the method of choice for the purification of very large plasmids (> 15 kb), which are vulnerable to nicking, and closed circular plasmids that are to be used for biophysical measurements. However, both methods of purification yield plasmid DNAs that can be used for a variety of sophisticated tasks in molecular cloning, including transfection of mammalian cells and the generation of sets of deletion mutants with exonucleases For more routine procedures, the problem of extensive purification can fortunately be avoided altogether Most of the plasmids in common use replicate so profusely that sufficient DNA can be obtained from minipreps to accomplish such tasks as the construction of restriction maps, transformation of bacteria, isolation of specific DNA fragments, routine subcloning, and generation of radiolabeled probes

SMALL-SCALE PREPARATIONS OF PLASMID DNA

Minipreparations of plasmid DNA can be obtained either by the alkaline lysis method presented below or by the boiling method (see page 1.29).

Harvesting and Lysis of Bacteria

HARVESTING

1. Transfer a single bacterial colony into 2 ml of LB medium containing the appropriate antibiotic in a loosely capped 15-ml tube. Incubate the culture overnight at 37°C with vigorous shaking

2. Pour 1.5 ml of the culture into a microfuge tube Centrifuge at 12,000*g* for 30 seconds at 4°C in a microfuge Store the remainder of the culture at 4°C

3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible

   The supernatant can be conveniently removed with a disposable pipette tip attached to a vacuum line (see Figure 1.3) Use a gentle vacuum and touch the tip to the surface of the liquid Keep the tip as far away from the bacterial pellet as possible as the fluid is withdrawn from the tube The pipette tip can then be used to vacuum the walls of the tube to remove any adherent droplets of fluid

LYSIS BY ALKALI

This protocol is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

1. Resuspend the bacterial pellet (obtained in step 3 above) in 100 μl of ice-cold Solution I by vigorous vortexing

   Solution I

   50 mM glucose
   25 mM Tris·Cl (pH 8.0)
   10 mM EDTA (pH 8.0)

   Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 10 lb/sq. in. on liquid cycle, and stored at 4°C.

   It is essential to ensure that the bacterial pellet is completely dispersed in Solution I This can be achieved rapidly by vortexing two microfuge tubes simultaneously with their bases touching as shown in Figure 1.2.

   The original protocol called for the use of lysozyme at this point to digest the bacterial cells. This step is unnecessary

   Some strains of bacteria shed into the medium cell-wall components that can inhibit the action of restriction enzymes. This problem can be avoided by resuspending the bacterial cell pellet in 0.5 ml of STE (0.1 M NaCl, 10 mM Tris Cl [pH 8.0], 1 mM EDTA [pH 8.0]) and recentrifuging After removal of the STE, resuspend the pellet in Solution I as described above

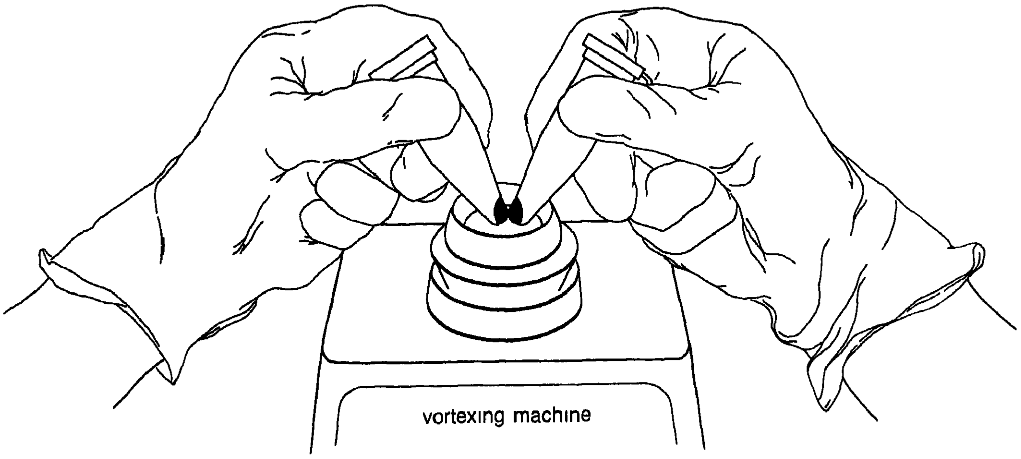


FIGURE 1.2
Vortexing to disperse bacterial pellet

2. Add 200 μl of freshly prepared Solution II

   Solution II

   0.2 N NaOH (freshly diluted from a 10 N stock)
   1% SDS

   Close the tube tightly, and mix the contents by inverting the tube rapidly five times Make sure that the entire surface of the tube comes in contact with Solution II Do not vortex Store the tube on ice

3. Add 150 μl of ice-cold Solution III

   Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

   The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

   Close the tube and vortex it gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate Store the tube on ice for 3–5 minutes.

4. Centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge Transfer the supernatant to a fresh tube.

5. *Optional.* Add an equal volume of phenol:chloroform Mix by vortexing. After centrifuging at 12,000*g* for 2 minutes at 4°C in a microfuge, transfer the supernatant to a fresh tube.

   Some workers find the extraction with phenol chloroform to be unnecessary However, for reasons that are unknown, the elimination of this step often results in DNA that is resistant to cleavage by restriction enzymes

6. Precipitate the double-stranded DNA with 2 volumes of ethanol at room temperature Mix by vortexing. Allow the mixture to stand for 2 minutes at room temperature.

7. Centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

8. Remove the supernatant by gentle aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away Remove any drops of fluid adhering to the walls of the tube.

   The supernatant can be conveniently removed with a disposable pipette tip attached to a vacuum line (Figure 1 3) Use a gentle vacuum and touch the tip to the surface

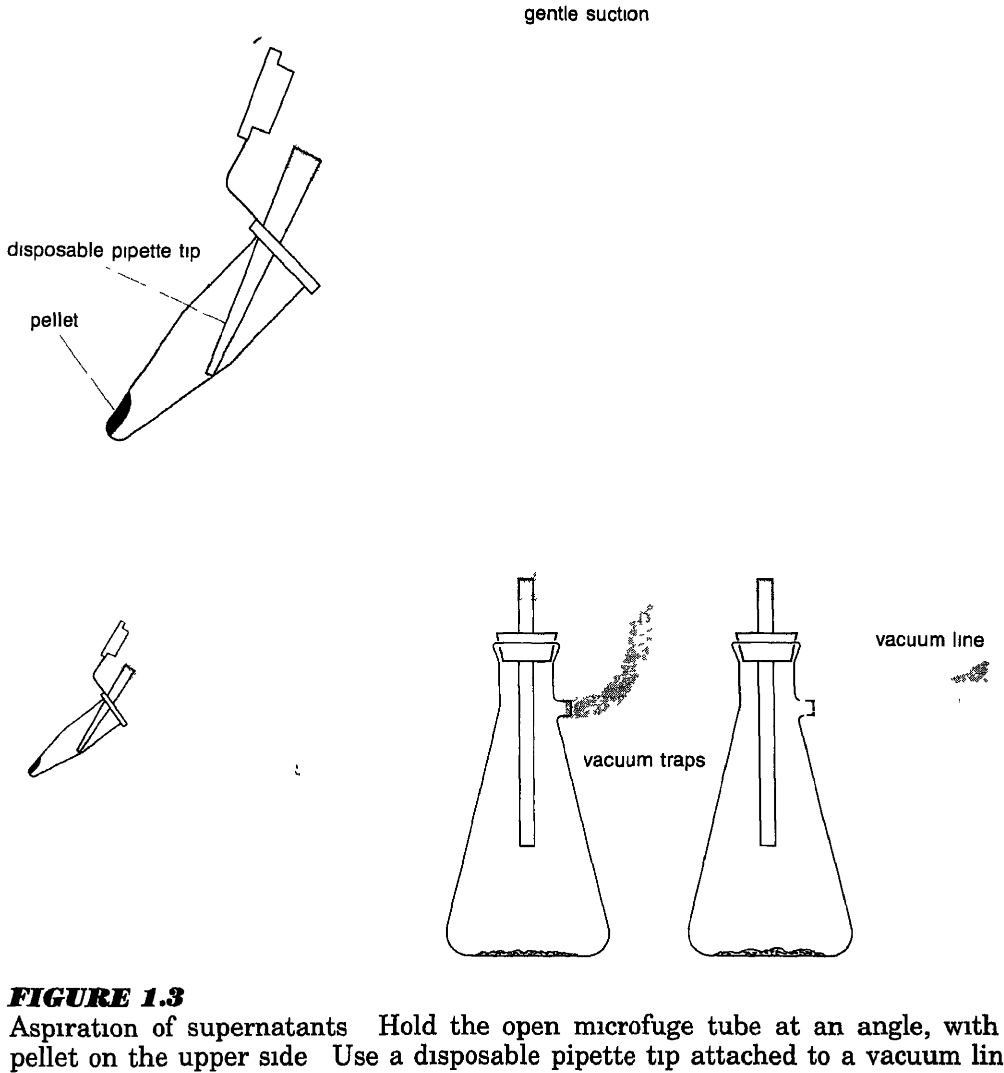


FIGURE 1.3
Aspiration of supernatants Hold the open microfuge tube at an angle, with the pellet on the upper side Use a disposable pipette tip attached to a vacuum line to withdraw fluid from the tube Insert the tip just beneath the meniscus on the lower side of the tube Move the tip towards the base of the tube as the fluid is withdrawn. Use gentle suction to avoid drawing the pellet into the pipette tip. Keep the end of the tip away from the pellet Finally, vacuum the walls of the tube to remove any adherent drops of fluid

of the liquid Keep the tip as far away from the pellet of nucleic acid as possible as the fluid is withdrawn from the tube The pipette tip can then be used to vacuum the walls of the tube to remove any adherent droplets of fluid

9. Rinse the pellet of double-stranded DNA with 1 ml of 70% ethanol at 4°C Remove the supernatant as described in step 8, and allow the pellet of nucleic acid to dry in the air for 10 minutes

10. Redissolve the nucleic acids in 50 μl of TE (pH 8 0) containing DNAase-free pancreatic RNAase (20 μg/ml) (see Appendix B) Vortex briefly Store the DNA at −20°C

Notes

i The typical yield of high-copy-number plasmids, such as Xf3 or pUC, prepared by this method is about 3–5 μg of DNA per milliliter of original bacterial culture

ii To analyze the DNA by cleavage with restriction enzyme(s), remove 1 μl of the DNA solution and add it to a fresh microfuge tube that contains 8 μl of water Add 1 μl of the appropriate 10 × restriction enzyme buffer and 1 unit of the desired restriction enzyme Incubate the reaction for 1–2 hours at the appropriate temperature Store the remainder of the DNA preparation at −20°C Analyze the DNA fragments in the restriction digest by gel electrophoresis

If the miniprep DNA is resistant to cleavage with the restriction enzyme, it is likely that insufficient care was taken to remove all of the fluid at step 3, page 1 25, or step 8 above In this case, extract the final DNA preparation with phenol chloroform and reprecipitate the DNA with ethanol

Frequently, difficulties in cleavage with restriction enzymes can be overcome by digesting in a volume of 100–200 μl and using five- to tenfold more enzyme (especially for inexpensive enzymes) After digestion, add 0 1 volume of 3 M sodium acetate (pH 5 2) and precipitate the DNA with 2 volumes of ethanol

iii The protocol can be modified to accommodate up to 10 ml of bacterial culture as follows

a Centrifuge 10 ml of bacterial culture at 4000 rpm for 10 minutes at 4°C in a Sorvall SS34 rotor (or its equivalent)

b At step 1, resuspend the bacterial pellet in 200 μl of Solution I and transfer the suspension to a microfuge tube

c At step 2, add 400 μl of Solution II

d At step 3, add 300 μl of ice-cold Solution III

e At step 5, transfer 600 μl of the supernatant to a fresh tube.

f At step 6, precipitate the DNA with 600 μl of isopropanol.

The yield of DNA is often sufficient to carry out the next step in a cloning protocol without going to the trouble or expense of a large-scale plasmid preparation

LYSIS BY BOILING

This method is adapted from Holmes and Quigley (1981).

1. Resuspend the bacterial pellet (obtained in step 3, page 1.25) in 350 μl of STET.

   STET

   0.1 M NaCl
   10 mM Tris · Cl (pH 8.0)
   1 mM EDTA (pH 8.0)
   5% Triton X-100

2. Add 25 μl of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris · Cl [pH 8.0]). Mix by vortexing for 3 seconds.

   Lysozyme will not work efficiently if the pH of the solution is less than 8.0.

3. Place the tube in a boiling-water bath for exactly 40 seconds.

4. Centrifuge the bacterial lysate at 12,000*g* for 10 minutes at room temperature in a microfuge.

5. Remove the pellet of bacterial debris from the microfuge tube with a sterile toothpick.

6. Add to the supernatant 40 μl of 2.5 M sodium acetate (pH 5.2) and 420 μl of isopropanol. Mix by vortexing, and store the tube for 5 minutes at room temperature.

7. Recover the pellet of nucleic acids by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

8. Remove the supernatant by gentle aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube.

   The supernatant can be conveniently removed with a disposable pipette tip attached to a vacuum line (see Figure 1.3). Use a gentle vacuum and touch the tip to the surface of the liquid. Keep the tip as far away from the pellet of nucleic acid as possible while the fluid is withdrawn from the tube. The pipette tip can then be used to vacuum the walls of the tube to remove any adherent droplets of fluid.

9. Add 1 ml of 70% ethanol and recentrifuge at 12,000*g* for 2 minutes at 4°C in a microfuge.

10. Again remove all of the supernatant by gentle aspiration as described in step 8. Take care with this step, as the pellet sometimes does not adhere tightly to the tube. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (2–5 minutes).

11. Redissolve the nucleic acids in 50 μl of TE (pH 8 0) containing DNAase-free pancreatic RNAase (20 μg/ml) (see Appendix B) Vortex briefly Store the DNA at −20°C.

Notes

i. The boiling method is not recommended when making small-scale preparations of plasmid DNA from strains of *E. coli* that express endonuclease A (*endA*$^+$ strains; e.g., HB101) Because endonuclease A is not completely inactivated by the boiling procedure, the plasmid DNA is degraded during subsequent incubation in the presence of Mg^{++} (e g , during digestion with restriction enzymes). This problem can be avoided by including an extra step—extraction with phenol:chloroform—in the protocol between steps 8 and 9

ii The typical yield of high-copy-number plasmids, such as Xf3 or pUC, prepared by this method is about 3–5 μg of DNA per milliliter of original bacterial culture

iii To analyze the DNA by cleavage with restriction enzyme(s), remove 1 μl of the DNA solution and add it to a fresh microfuge tube that contains 8 μl of water. Add 1 μl of the appropriate 10 × restriction enzyme buffer and 1 unit of the desired restriction enzyme Incubate the reaction for 1–2 hours at the appropriate temperature Store the remainder of the DNA preparation at −20°C Analyze the DNA fragments in the restriction digest by gel electrophoresis.

If the miniprep DNA is resistant to cleavage with the restriction enzyme, it is likely that insufficient care was taken to remove all of the fluid at step 3, page 1 25, or step 8 above In this case, extract the final DNA preparation with phenol chloroform and reprecipitate the DNA with ethanol

Frequently, difficulties in cleavage with restriction enzymes can be overcome by digesting in a volume of 100–200 μl and using five- to tenfold more enzyme (especially for inexpensive enzymes) After digestion, add 0.1 volume of 3 M sodium acetate (pH 5 2) and precipitate the DNA with 2 volumes of ethanol

Solving Problems That Arise with Minipreparations of Plasmid DNA

Both the alkaline lysis and boiling methods are extremely reliable, highly reproducible, and generally free of trouble. In all the years that these methods have been in common use in our laboratories, only two problems have arisen.

- When workers make minipreps for the first time, the plasmid DNA sometimes is resistant to cleavage by restriction enzymes. Almost always, this problem occurs because insufficient care is taken to remove all of the supernatant fluid from the pellet of bacteria or from the precipitates of nucleic acid In most cases, the impurities in the miniprep can be removed by extracting the solution with phenol:chloroform and precipitating the DNA with ethanol If problems still persist, the DNA can be purified by spun-column chromatography (see Appendix E).
- Very occasionally, individual minipreps are found to contain no plasmid DNA This almost always occurs because the pellet of nucleic acid has been discarded with the ethanol

Rapid Disruption of Bacterial Colonies to Test the Size of Plasmids

Frequently, it is possible to determine from the size of a plasmid whether or not it carries an insert. The protocol described below (devised by J. Jenkins [pers. comm.] and based on the method of Barnes [1977]) yields enough DNA to load on a single lane of an agarose gel.

1. Grow bacterial colonies on rich agar medium (LB or SOB) containing the appropriate antibiotic until they are approximately 1 mm in diameter.

2. Using a sterile toothpick or disposable loop, transfer a small segment of a bacterial colony to a streak or patch on a master agar plate containing the appropriate antibiotic. When all of the colonies have been replicated in this fashion, incubate the master plate for several hours at 37°C and then store it at 4°C until it is needed to recover the appropriate colonies.

3. Transfer the remainder of each colony from the original plate to a microfuge tube containing 50 μl of a sterile solution of 10 mM EDTA (pH 8.0).

4. Add 50 μl of a freshly made solution of 0.2 N NaOH, 0.5% SDS, 20% sucrose. Vortex the mixture for 30 seconds.

5. Incubate the mixture for 5 minutes at 70°C, and then allow it to cool to room temperature.

6. Add 1.5 μl of a solution of 4 M KCl and 0.5 μl of a solution containing 0.4% bromophenol blue. Vortex the mixture for 30 seconds.

7. Incubate the mixture for 5 minutes on ice.

8. Remove bacterial debris by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge.

9. Load 50 μl of the supernatant into a slot (5 mm in length × 2.5 mm in width) cast in a 0.7% agarose gel (5 mm thick).

10. After the dye has migrated two thirds to three fourths the length of the gel, stain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0.5 μg/ml in water) at room temperature.

    The rate of migration of superhelical DNA in the absence of ethidium bromide reflects its molecular weight more faithfully than its rate of migration in the presence of the intercalating dye.

    Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 1.49–1.50.

LARGE-SCALE PREPARATIONS OF PLASMID DNA

Amplification of Plasmids in Rich Medium

For many years, it was thought that amplification of plasmids in the presence of chloramphenicol was effective only when the host bacteria were grown in minimal medium. However, the following procedure gives reproducibly high yields (2–5 mg of plasmid DNA per 500 ml of culture) with strains of *E. coli* harboring high-copy-number plasmids carrying the pMB1 or ColE1 replicon.

1. Grow a 30-ml culture of the bacterial strain carrying the plasmid of interest to late log phase (i.e., to an OD_{600} of ~0.6).

   The medium should be inoculated with a single bacterial colony or with a small liquid culture grown from a single colony. The appropriate antibiotic should be present in the medium.

2. Inoculate 500 ml of LB or Terrific Broth medium (prewarmed to 37°C) containing the appropriate antibiotic in a 2-liter flask with 25 ml of the late-log-phase culture. Incubate the culture for exactly 2.5 hours at 37°C with vigorous shaking (300 cycles/minute on a rotary shaker). The OD_{600} of the resulting culture will be ~0.4.

   To confirm that the culture contains the correct plasmid, prepare a small amount of plasmid DNA by one of the methods described on pages 1.25–1.28 and 1.29–1.30 and analyze it by digestion with the appropriate restriction enzyme(s).

3. *Optional:* Add 2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol). The final concentration of chloramphenicol in the culture is 170 μg/ml.

   Amplification is necessary to achieve high yields of plasmids, such as pBR322, that replicate to only moderate copy numbers in their host bacteria. Plasmids of a later generation (e.g., pUC plasmids) replicate to such high copy numbers that amplification is unnecessary. These plasmids can be purified in large yield from bacterial cultures that are allowed to grow to saturation. However, treatment with chloramphenicol has the advantage of inhibiting bacterial replication. This reduces the bulk and viscosity of the bacterial lysate and greatly simplifies purification of the plasmid. In general, the benefits of chloramphenicol treatment outweigh the small inconvenience involved in adding the drug to the growing bacterial culture.

4. Incubate the culture for a further 12–16 hours at 37°C with vigorous shaking (300 cycles/minute on a rotary shaker).

Harvesting and Lysis of Bacteria

HARVESTING

1. Harvest the bacterial cells from a 500-ml culture by centrifugation at 4000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor (or its equivalent) Discard the supernatant. Stand the open centrifuge bottle in an inverted position to allow all of the supernatant to drain away

2. Resuspend the bacterial pellet in 100 ml of ice-cold STE

   STE

   0.1 M NaCl
   10 mM Tris·Cl (pH 8.0)
   1 mM EDTA (pH 8.0)

3. Collect the bacterial cells by centrifugation as described in step 1

LYSIS BY BOILING

This method (adapted from Holmes and Quigley 1981) is designed to be used in conjunction with a subsequent purification step, e g , equilibrium centrifugation in CsCl–ethidium bromide gradients It is recommended only for cultures that have been treated with chloramphenicol Untreated cultures produce lysates that are too viscous to be manageable

Lysis by boiling should not be used when making large-scale preparations of plasmids from *E coli* strain HB101 and its derivatives (which include TG1) These bacteria release a large amount of carbohydrate that bands at approximately the same density in CsCl–ethidium bromide gradients as closed circular plasmid DNA The carbohydrate inhibits enzymes that use DNA as a template or substrate

1. Resuspend the washed bacterial pellet from a 500-ml culture (obtained in step 3 above) in 10 ml of ice-cold STET

   STET

   0.1 M NaCl
   10 mM Tris·Cl (pH 8.0)
   1 mM EDTA (pH 8.0)
   5% Triton X-100

   Transfer the suspension to a 50-ml Erlenmeyer flask

2. Add 1 ml of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris·Cl [pH 8 0]).

   Lysozyme will not work efficiently if the pH of the solution is less than 8 0

3. Using a clamp, hold the Erlenmeyer flask over the open flame of a Bunsen burner until the liquid *just* starts to boil Shake the flask constantly.

4. Immediately immerse the flask in a large (2-liter) beaker of boiling water. Hold the flask in the boiling water for exactly 40 seconds.

5. Cool the flask by immersing it in ice-cold water for 5 minutes

6. Transfer the viscous contents of the flask to an ultracentrifuge tube (Beckman SW41 or its equivalent) Centrifuge at 30,000 rpm for 30 minutes at 4°C

   The denser the growth of the original bacterial culture, the more difficult it is to transfer the viscous lysate to the centrifuge tube If absolutely necessary, the lysate can be chopped into chunks of manageable size with a pair of long-blade scissors, or it can be partially sheared by drawing it into the barrel of a 10-ml syringe This problem generally does not arise when isolating plasmid DNA from bacterial cultures that have been treated with chloramphenicol

   If the bacterial debris does not form a tightly packed pellet, recentrifuge at 35,000 rpm for a further 20 minutes, and then transfer as much of the supernatant as possible to a new tube Discard the viscous liquid remaining in the centrifuge tube

7. Transfer the supernatant to a fresh tube, and purify the plasmid DNA by equilibrium centrifugation in CsCl–ethidium bromide gradients (see page 1 42)

LYSIS BY SODIUM DODECYL SULFATE

This method (adapted from Godson and Vapnek 1973) gives much lower yields but is considerably more gentle than those described elsewhere in this chapter It is the method of choice for isolating large plasmids (> 15 kb)

1. Resuspend the washed bacterial pellet from a 500-ml culture (obtained in step 3, page 1 34) in 10 ml of an ice-cold solution of 10% sucrose in 50 mM Tris · Cl (pH 8 0). Transfer the suspension to a 30-ml plastic screw-cap tube (Oak Ridge style).

2. Add 2 ml of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris Cl [pH 8.0])

   Lysozyme will not work efficiently if the pH of the solution is less than 8 0

3. Add 8 ml of 0.25 M EDTA (pH 8 0) Mix the suspension by inverting the tube several times. Place the tube on ice for 10 minutes

4. Add 4 ml of 10% SDS *Immediately* mix the contents of the tube with a glass rod so as to disperse the SDS evenly throughout the bacterial suspension Be as gentle as possible to minimize shearing of the liberated DNA

5. As soon as mixing is completed, add 6 ml of 5 M NaCl (final concentration = 1 M) Again, use a glass rod to mix the contents of the tube gently but thoroughly Place the tube on ice for at least 1 hour

6. Remove high-molecular-weight DNA and bacterial debris by centrifugation at 30,000 rpm for 30 minutes at 4°C in a Beckman Ti50 rotor (or its equivalent) Carefully transfer the supernatant to a 50-ml disposable plastic centrifuge tube Discard the pellet

   If the bacterial debris does not form a tightly packed pellet, recentrifuge at 35,000 rpm for a further 20 minutes, and then transfer as much of the supernatant as possible to a fresh tube Discard the viscous liquid remaining in the centrifuge tube

7. Extract the supernatant once with phenol.chloroform and once with chloroform.

8. Transfer the aqueous phase to a 250-ml centrifuge bottle Add 2 volumes (~ 60 ml) of ethanol at room temperature Mix well Let stand at room temperature for 1–2 hours

9. Recover the nucleic acids by centrifugation at 5000*g* for 20 minutes at 4°C.

   Horizontal rotors are better than angle rotors for this step, since they concentrate the nucleic acids on the bottom of the bottle rather than smearing them on the walls

10. Discard the supernatant Wash the pellet with 70% ethanol at room temperature and recentrifuge as in step 9 Discard as much of the ethanol as possible, and then invert the centrifuge bottle on a pad of paper towels to allow the last of the ethanol to drain away Dry the centrifuge bottle briefly in a vacuum desiccator, but do not allow the pellet to dry completely

11. Dissolve the DNA in 3 ml of TE (pH 8.0)

12. Purify the plasmid DNA by equilibrium centrifugation in CsCl–ethidium bromide gradients (see page 1 42)

Large plasmids (> 15 kb) are vulnerable to nicking during the several precipitation steps that are used in purification by polyethylene glycol Purification by equilibrium centrifugation in CsCl–ethidium bromide gradients is therefore the method of choice with such plasmids

LYSIS BY ALKALI

This protocol is an unpublished procedure of R. Treisman and is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). The method, which works well with all commonly used strains of *E. coli,* is designed to be used in conjunction with a subsequent purification step, such as precipitation with polyethylene glycol or equilibrium centrifugation in CsCl–ethidium bromide gradients.

Note The volumes given in parentheses should be used with cultures that have not been treated with chloramphenicol.

1. Resuspend the washed bacterial pellet from a 500-ml culture (obtained in step 3, page 1.34) in 10 ml (18 ml) of Solution I.

Solution I

50 mM glucose
25 mM Tris · Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of ~100 ml, autoclaved for 15 minutes at 10 lb/sq in. on liquid cycle, and stored at 4°C.

2. Add 1 ml (2 ml) of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris · Cl [pH 8.0]).

Lysozyme will not work efficiently if the pH of the solution is less than 8.0.

3. Add 20 ml (40 ml) of freshly prepared Solution II.

Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)
1% SDS

Close the top of the centrifuge bottle and mix the contents thoroughly by gently inverting the bottle several times. Store the bottle at room temperature for 5–10 minutes.

4. Add 15 ml (20 ml) of ice-cold Solution III.

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Close the top of the centrifuge bottle and mix the contents by shaking the bottle several times There should no longer be two distinguishable liquid phases Store the bottle on ice for 10 minutes. A flocculent white precipitate should form

The precipitate that forms during storage at 0°C consists of chromosomal DNA, high-molecular-weight RNA, and potassium/SDS/protein/membrane complexes

5. Centrifuge the bacterial lysate at 4000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor (or its equivalent). Allow the rotor to stop without braking

   If the bacterial debris does not form a tightly packed pellet, recentrifuge at 5000 rpm for a further 20 minutes, and then transfer as much of the supernatant as possible to a fresh bottle Discard the viscous liquid remaining in the centrifuge bottle The failure to form a compact pellet is usually a consequence of inadequate mixing of the bacterial lysate with Solution III (step 4)

6. Filter the supernatant through four layers of cheesecloth into a 250-ml centrifuge bottle Add 0 6 volume of isopropanol, mix well, and store the bottle for 10 minutes at room temperature

7. Recover the nucleic acids by centrifugation at 5000 rpm for 15 minutes at room temperature in a Sorvall GS3 rotor (or its equivalent)

   Salt may precipitate if centrifugation is carried out at 4°C

8. Decant the supernatant carefully, and invert the open bottle to allow the last drops of supernatant to drain away. Rinse the pellet and the walls of the bottle with 70% ethanol at room temperature Drain off the ethanol, and use a pasteur pipette attached to a vacuum line to remove any beads of liquid that adhere to the walls of the bottle Place the inverted, open bottle on a pad of paper towels for a few minutes at room temperature to allow the final traces of ethanol to evaporate.

9. Dissolve the pellet of nucleic acid in 3 ml of TE (pH 8 0).

10. Purify the plasmid DNA either by equilibrium centrifugation in CsCl–ethidium bromide gradients (see page 1 42) or by precipitation with polyethylene glycol (see page 1 40)

PURIFICATION OF PLASMID DNA

Purification of Plasmid DNA by Precipitation with Polyethylene Glycol

The method presented here (R. Treisman, pers. comm) has been used effectively to purify plasmid DNA prepared by the alkaline lysis method

1. Transfer the nucleic acid solution (from step 9, page 1 39) to a 15-ml Corex tube, and add 3 ml of an ice-cold solution of 5 M LiCl Mix well, and then centrifuge the solution at 10,000 rpm for 10 minutes at 4°C in a Sorvall SS34 rotor (or its equivalent)

   LiCl precipitates high-molecular-weight RNA

2. Transfer the supernatant to a fresh 30-ml Corex tube Add an equal volume of isopropanol. Mix well Recover the precipitated nucleic acids by centrifugation at 10,000 rpm for 10 minutes at room temperature in a Sorvall SS34 rotor (or its equivalent)

3. Decant the supernatant carefully, and invert the open tube to allow the last drops of supernatant to drain away Rinse the pellet and the walls of the tube with 70% ethanol at room temperature Drain off the ethanol, and use a pasteur pipette attached to a vacuum line to remove any beads of liquid that adhere to the walls of the tube Place the inverted, open tube on a pad of paper towels for a few minutes at room temperature to allow the last traces of ethanol to evaporate

4. Dissolve the pellet in 500 μl of TE (pH 8 0) containing DNAase-free pancreatic RNAase (20 μg/ml) (see Appendix B) Transfer the solution to a microfuge tube and store at room temperature for 30 minutes

5. Add 500 μl of 1 6 M NaCl containing 13% (w/v) polyethylene glycol (PEG 8000) Mix well Recover the plasmid DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge

6. Remove the supernatant by aspiration Dissolve the pellet of plasmid DNA in 400 μl of TE (pH 8 0) Extract the solution once with phenol, once with phenol.chloroform, and once with chloroform

7. Transfer the aqueous phase to a fresh microfuge tube Add 100 μl of 10 M ammonium acetate, and mix well Add 2 volumes (~1 ml) of ethanol, and store the tube for 10 minutes at room temperature. Recover the precipitated plasmid DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge

8. Remove the supernatant by aspiration Add 200 μl of 70% ethanol at 4°C. Vortex briefly, and then centrifuge at 12,000*g* for 2 minutes at 4°C in a microfuge.

9. Remove the supernatant by aspiration, and store the open tube on the bench until the last visible traces of ethanol have evaporated.

10. Dissolve the pellet in 500 μl of TE (pH 8.0). Measure the OD$_{260}$ of a 1.100 dilution (in TE [pH 8.0]) of the solution, and calculate the concentration of the plasmid DNA (1 OD$_{260}$ = 50 μg of plasmid DNA/ml). Store the DNA in aliquots at −20°C.

Purification of Closed Circular DNA by Equilibrium Centrifugation in CsCl–Ethidium Bromide Gradients

CONTINUOUS GRADIENTS

1. Measure the volume of the DNA solution. For every milliliter, add exactly 1 g of solid CsCl. Warm the solution to 30°C to facilitate the dissolution of the salt. Mix the solution gently until the salt is dissolved.

2. Add 0.8 ml of a solution of ethidium bromide (10 mg/ml in water) for every 10 ml of the DNA/CsCl solution. Immediately mix the ethidium bromide solution (which floats on the surface) with the denser DNA/CsCl solution. The final density of the solution should be 1.55 g/ml (refractive index = 1.3860), and the concentration of ethidium bromide should be approximately 740 μg/ml.

   Stock solutions of ethidium bromide should be stored in light-tight containers (e.g., in a bottle completely wrapped in aluminum foil) at room temperature.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 1.49–1.50.

3. Centrifuge the solution at 8000 rpm for 5 minutes at room temperature in a Sorvall SS34 rotor (or its equivalent). The furry scum that floats to the top consists of complexes formed between the ethidium bromide and bacterial proteins.

4. Using a pasteur pipette or a disposable syringe fitted with a large-gauge needle, transfer the clear, red solution under the scum to a tube (Beckman Quick-Seal or equivalent) suitable for centrifugation in a Beckman vertical Ti65 rotor or an angle Ti50, Ti65, or Ti70 rotor (or their equivalents). Fill the remainder of the tube with light paraffin oil and seal the tube.

5. Centrifuge the density gradients at 45,000 rpm for 16 hours (VTi65), 45,000 rpm for 48 hours (Ti50), 60,000 rpm for 24 hours (Ti65), or 60,000 rpm for 24 hours (Ti70.1) at 20°C.

   Two bands of DNA, located in the center of the gradient, should be visible in ordinary light (see Figure 1.4, page 1.45). The upper band, which usually contains less material, consists of linear bacterial (chromosomal) DNA and nicked circular plasmid DNA; the lower band consists of closed circular plasmid DNA. The deep-red pellet on the bottom of the tube consists of ethidium bromide/RNA complexes. The material between the CsCl solution and the paraffin oil is protein.

   CsCl–ethidium bromide gradients in Beckman Quick-Seal tubes can accommodate up to 4 mg of closed circular plasmid DNA without becoming overloaded. If more plasmid DNA is present, it spreads into a wide band that overlaps with the chromosomal DNA. This problem, which arises only with plasmids that replicate to extremely high levels, can be avoided by dividing such plasmid preparations between two gradients. If overloading does occur, collect the entire band of DNA, adjust the volume of the solution to 15 ml with CsCl solution ($\rho = 1.58$ g/ml), and recentrifuge the DNA to equilibrium in two centrifuge tubes

6. Collect the bands of DNA as shown in Figure 1 4 (page 1.45) and described here Insert a 21-gauge hypodermic needle into the top of the tube to allow air to enter. To minimize the possibility of contamination, first collect the upper band (chromosomal DNA) through an 18-gauge hypodermic needle as follows: Carefully wipe the outside of the tube with ethanol to remove any grease or oil, and then attach a piece of Scotch Tape to the outside of the tube. Insert an 18-gauge hypodermic needle (beveled side up) into the tube through the tape so that the open, beveled side of the needle is positioned just below the band of chromosomal DNA and parallel to it Collect the viscous DNA into a disposable tube, and then seal the end of the hypodermic needle with a plug of modeling clay. Leaving the second needle in place, insert a third hypodermic needle (18 gauge) through the tape and collect the lower band of plasmid DNA into a glass or plastic tube.

7. Remove ethidium bromide from the DNA as described on page 1 46 or 1 47

DISCONTINUOUS GRADIENTS

In this method, solutions containing different concentrations of CsCl are layered into the centrifuge tube This speeds the formation of the CsCl gradient and allows the centrifugation time to be reduced to 6 hours

1. Prepare a solution of CsCl ($\rho = 1\ 47$ g/ml) by adding 125 g of CsCl to 167 ml of TE (pH 8.0).
2. Add 8 ml of the CsCl solution to a Beckman Quick-Seal centrifuge tube (or its equivalent). Put this aside until needed in step 8
3. If necessary, adjust the volume of the solution of plasmid DNA to exactly 3 ml with TE (pH 8 0).
4. Add 8.4 g of CsCl to the solution of plasmid DNA Warm the solution to 30°C to facilitate dissolution of the salt Mix the solution gently until the salt is dissolved
5. Weigh the solution and add TE (pH 8 0) until the solution weighs exactly 13 2 g Be certain to tare the balance with the weight of the tube
6. Add 0.8 ml of a solution of ethidium bromide (10 mg/ml in water) Quickly mix the solution until the dye is homogeneously dispersed The volume of the solution should now be approximately 7 5 ml

   Stock solutions of ethidium bromide should be stored in light-tight containers (e g , in a bottle completely wrapped in aluminum foil) at room temperature

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described on pages 1 49–1.50
7. Centrifuge the solution at 8000 rpm for 5 minutes at room temperature in a Sorvall SS34 rotor (or its equivalent) The furry scum that floats to the top consists of complexes formed between the ethidium bromide and bacterial proteins.
8. Place a 9-inch pasteur pipette into the centrifuge tube containing CsCl prepared in step 2 The tip of the pipette should touch the bottom of the tube Carefully load the clear, red solution (from beneath the scum) prepared in step 7 into the tube through the pipette so that the sample is layered under the 1 47 g/ml CsCl solution already in the tube If necessary, fill the tube with the CsCl solution ($\rho = 1\ 47$ g/ml) prepared in step 1. Seal the tube.
9. Load the sealed tube (together with an appropriate balancing tube) into a Beckman Ti70.1 or Sorvall 65 13 rotor (or equivalent), and centrifuge the density gradient at 60,000 rpm for 6 hours at 20°C.
10. Recover the band of closed circular plasmid DNA as described in step 6, page 1.43, and shown in Figure 1.4
11. Remove ethidium bromide from the DNA as described on page 1.46 or 1.47.

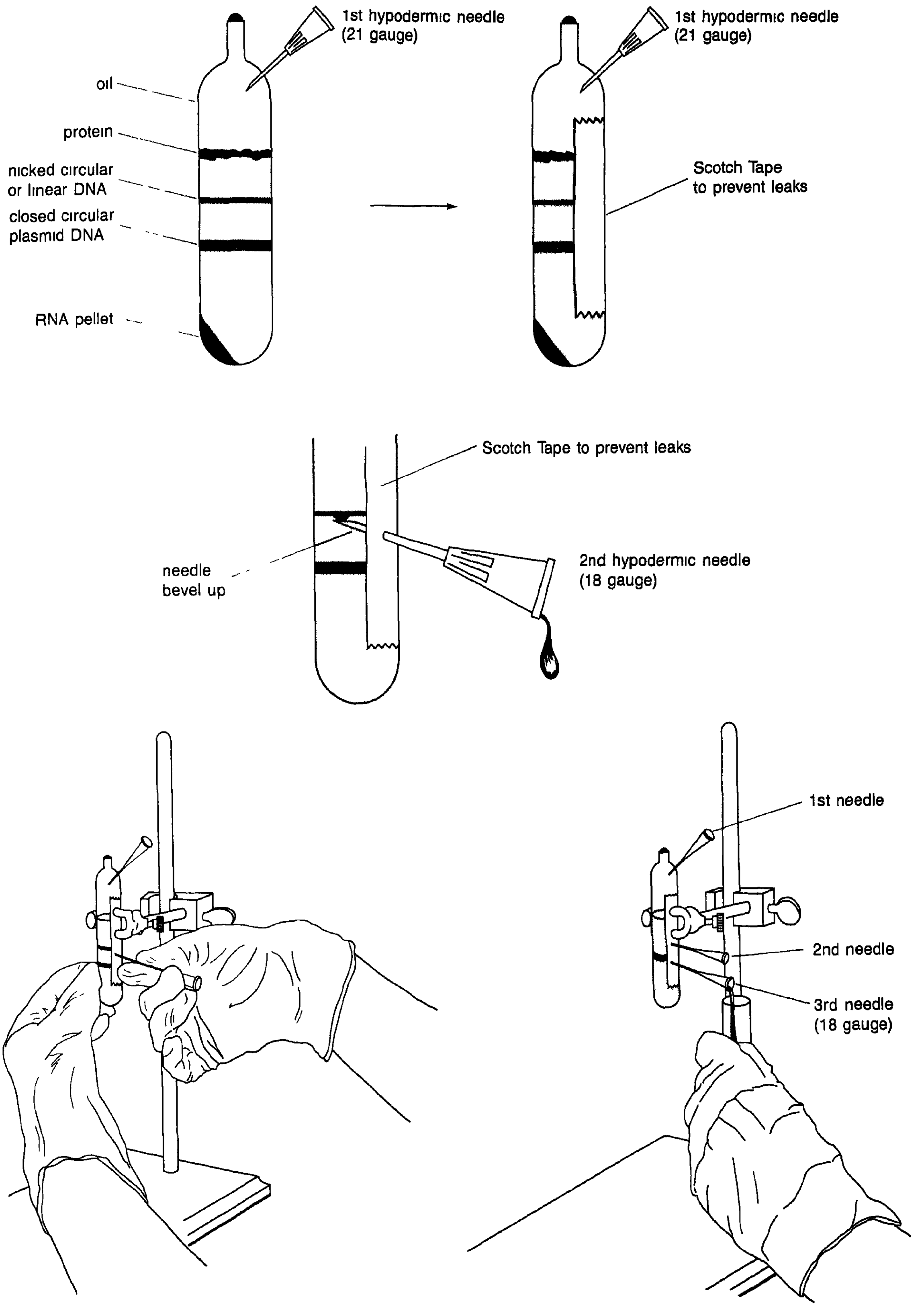


FIGURE 1.4
Collection of superhelical plasmid DNA from CsCl gradients containing ethidium bromide. See step 6, page 1.43, for details.

Removal of Ethidium Bromide from DNAs Purified by Equilibrium Centrifugation in CsCl–Ethidium Bromide Gradients

Either of the two methods given below are effective in removing ethidium bromide from DNA purified by equilibrium centrifugation in CsCl–ethidium bromide gradients. Method 1 is more commonly used

METHOD 1: EXTRACTION WITH ORGANIC SOLVENTS

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described on pages 1.49–1.50

1. To the solution of DNA in a glass or plastic tube, add an equal volume of *either* 1-butanol saturated with water *or* isoamyl alcohol

2. Mix the two phases by vortexing

3. Centrifuge the mixture at 1500 rpm for 3 minutes at room temperature in a bench centrifuge

4. Using a pasteur pipette, transfer the lower, aqueous phase to a clean glass or plastic tube.

5. Repeat the extraction (steps 1–4) four to six times until all the pink color disappears from both the aqueous phase and the organic phase

6. Remove the CsCl from the DNA solution either by spin dialysis through a microconcentrator (Amicon) (see Chapter 14, page 14 22), by dialysis for 24–48 hours against several changes of TE (pH 8 0), or by diluting with 3 volumes of water and precipitating the DNA with 2 volumes of ethanol (i e , the final volume equals six times the original volume of the undiluted aqueous phase) for 15 minutes at 4°C followed by centrifugation at 10,000*g* for 15 minutes at 4°C Dissolve the precipitated DNA in approximately 1 ml of TE (pH 8 0)

   CsCl precipitates if the ethanolic solution of DNA is stored at −20°C

7. Measure the OD_{260} of the final solution of DNA, and calculate the concentration of DNA (see Appendix E) Store the DNA in aliquots at −20°C.

Note

If the final preparation of DNA contains significant quantities of ethidium bromide, as judged from its color, extract it once with phenol and once with phenol:chloroform and then reprecipitate the DNA with ethanol.

METHOD 2: ION-EXCHANGE CHROMATOGRAPHY

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 1 49–1 50

1. Construct a 1-ml column of Dowex AG50 in a pasteur pipette as shown in Figure 1 5.

   Before use, Dowex AG50 resin should be equilibrated as follows

   a Stir approximately 20 g of Dowex AG50 in approximately 100 ml of 1 M NaCl for 5 minutes Allow the resin to settle, and remove the supernatant by aspiration

   b Add approximately 100 ml of 1 N HCl, and stir the slurry for a further 5 minutes Again allow the resin to settle, and remove the supernatant by aspiration

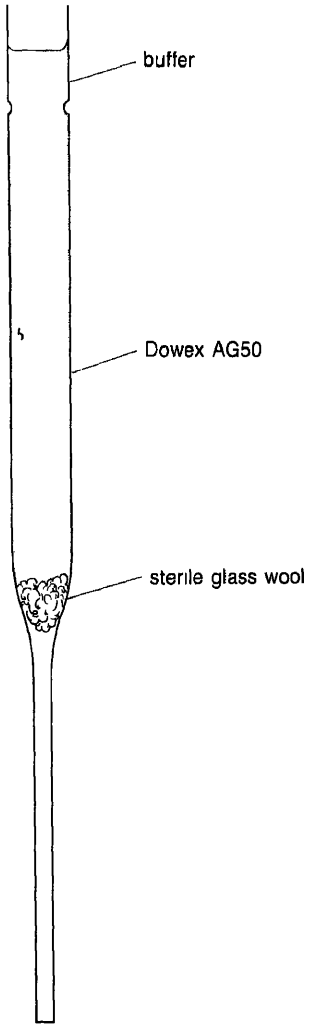


FIGURE 1.5
Removal of ethidium bromide from DNA by chromatography through Dowex AG50. See step 1 above for details.

c Repeat step b with two batches of water (100 ml each), followed by one batch of TEN buffer (100 ml)

TEN buffer

0.1 M Tris · Cl (pH 8.0)
0.01 M EDTA (pH 8.0)
1 M NaCl

d Store the resin at 4°C in TEN buffer containing 0 2% sodium azide

Caution: Sodium azide is poisonous It should be handled with great care wearing gloves, and solutions containing it should be clearly marked

2. Remove the buffer above the resin, and rinse the column with 2 volumes of TE (pH 8 0). Apply the solution of DNA containing ethidium bromide and CsCl directly to the resin

3. Immediately begin collecting the effluent from the column After all of the DNA solution has entered the column, wash the resin with 1 2 column volumes of TE (pH 8.0) and continue to collect the eluate

4. After the column has run dry, dilute the pooled eluate with 2 volumes of water

5. Precipitate the DNA with ethanol (use six times the original volume of the pooled eluate) for 15 minutes at 4°C Collect the DNA by centrifugation at 12,000 rpm for 15 minutes at 4°C in a Sorvall SS34 rotor (or its equivalent).

6. Remove the supernatant as completely as possible, and store the open tube for a few minutes in an inverted position on a pad of paper towels at room temperature to allow the last traces of ethanol to drain away

7. Wash the pellet with 70% ethanol at 4°C, and recentrifuge as in step 5 Remove the supernatant as completely as possible, and store the open tube for a few minutes in an inverted position on a pad of paper towels at room temperature to allow the last traces of ethanol to evaporate

8. Dissolve the DNA in approximately 1 ml of TE (pH 8 0) Measure the OD_{260} of the final solution of DNA, and calculate the concentration of DNA (see Appendix E). Store the DNA in aliquots at −20°C.

Note

If the final preparation of DNA contains significant quantities of ethidium bromide, as judged from its color, extract it once with phenol and once with phenol·chloroform and then reprecipitate the DNA with ethanol.

Decontamination of Ethidium Bromide Solutions

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic After use, solutions containing this dye should be decontaminated by one of the methods described below

DECONTAMINATION OF CONCENTRATED SOLUTIONS OF ETHIDIUM BROMIDE (i.e., solutions containing >0.5 mg/ml)

Method 1

This method (Lunn and Sansone 1987) reduces the mutagenic activity of ethidium bromide in the *Salmonella*/microsome assay by approximately 200-fold

1. Add sufficient water to reduce the concentration of ethidium bromide to <0.5 mg/ml
2. To the resulting solution, add 0.2 volume of fresh 5% hypophosphorous acid and 0.12 volume of fresh 0.5 M sodium nitrite Mix carefully
   Important: Check that the pH of the solution is <3.0

   Hypophosphorous acid is usually supplied as a 50% solution, which is corrosive and should be handled with care It should be freshly diluted immediately before use

   Sodium nitrite solution (0.5 M) should be freshly prepared by dissolving 34.5 g of sodium nitrite in water to a final volume of 500 ml
3. After incubation for 24 hours at room temperature, add a large excess of 1 M sodium bicarbonate The solution may now be discarded

Method 2

This method (Quillardet and Hofnung 1988) reduces the mutagenic activity of ethidium bromide in the *Salmonella*/microsome assay by approximately 3000-fold However, there are reports (Lunn and Sansone 1987) of mutagenic activity in occasional batches of "blanks" treated with the decontaminating solutions

1. Add sufficient water to reduce the concentration of ethidium bromide to <0.5 mg/ml
2. To the resulting solution, add 1 volume of 0.5 M $KMnO_4$ Mix carefully, and then add 1 volume of 2.5 N HCl Mix carefully, and allow the solution to stand at room temperature for several hours
3. Add 1 volume of 2.5 N NaOH Mix carefully, and then discard the solution.

DECONTAMINATION OF DILUTE SOLUTIONS OF ETHIDIUM BROMIDE

(e.g., electrophoresis buffer containing 0.5 μg/ml ethidium bromide)

Method 1

The following method is from Lunn and Sansone (1987)

1. Add 2.9 g of Amberlite XAD-16 for each 100 ml of solution Amberlite XAD-16, a nonionic, polymeric absorbent, is available from Rohm and Haas

2. Store the solution for 12 hours at room temperature, shaking it intermittently

3. Filter the solution through a Whatman No 1 filter, and discard the filtrate

4. Seal the filter and Amberlite resin in a plastic bag, and dispose of the bag in the hazardous waste

Method 2

The following method is from Bensaude (1988)

1. Add 100 mg of powdered activated charcoal for each 100 ml of solution

2. Store the solution for 1 hour at room temperature, shaking it intermittently

3. Filter the solution through a Whatman No 1 filter, and discard the filtrate

4. Seal the filter and activated charcoal in a plastic bag, and dispose of the bag in the hazardous waste.

Notes

i Treatment of dilute solutions of ethidium bromide with hypochlorite (bleach) is not recommended as a method of decontamination Such treatment reduces the mutagenic activity of ethidium bromide in the *Salmonella*/microsome assay by about 1000-fold, but it converts the dye into a compound that is mutagenic in the absence of microsomes (Quillardet and Hofnung 1988).

ii. Ethidium bromide decomposes at 262°C and is unlikely to be hazardous after incineration under standard conditions.

iii. Slurries of Amberlite XAD-16 or activated charcoal can be used to decontaminate surfaces that become contaminated by ethidium bromide.

Removal of RNA from Preparations of Plasmid DNA

For some purposes (e.g., for digestion with BAL 31 or for labeling the 5′ termini of restriction enzyme fragments of plasmid DNA with bacteriophage T4 polynucleotide kinase), it is essential to obtain DNA preparations that are free of contaminating RNA. Although the *weight* of such contaminants in plasmid DNA prepared by equilibrium centrifugation in CsCl–ethidium bromide gradients is small, the *number* of RNA molecules can be relatively large and can contribute significantly to the total number of 5′ termini in a restriction digest. RNA can be removed from plasmid preparations by the following two methods.

CENTRIFUGATION THROUGH 1 M NaCl

This is an unpublished protocol of B. Seed.

1. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and precipitate the nucleic acids with 2 volumes of ethanol for 30 minutes at 4°C.

2. Recover the nucleic acids by centrifugation at >10,000*g* for 15 minutes at 4°C. Drain off as much of the supernatant as possible, and then store the open tube on the bench to allow the last traces of ethanol to evaporate.

3. Dissolve the pellet in TE (pH 8.0) at a concentration of at least 100 μg/ml.

4. Add DNAase-free pancreatic RNAase (see Appendix B) to a final concentration of 10 μg/ml. Incubate the mixture for 1 hour at room temperature.

5. Add 4 ml of TE (pH 8.0) containing 1 M NaCl to a Beckman SW50.1 centrifuge tube (or its equivalent). Using an automatic pipetting device equipped with a disposable tip, layer up to 1 ml of the RNAase-treated plasmid preparation on top of the 1 M NaCl solution. If necessary, fill the tube with TE (pH 8.0). Centrifuge at 40,000 rpm for 6 hours at 20°C in a Beckman SW50.1 rotor (or its equivalent). The plasmid DNA sediments to the bottom of the tube while the ribo-oligonucleotides remain in the supernatant.

6. Discard the supernatant. Redissolve the pellet of plasmid DNA in 0.5 ml of TE (pH 8.0). Add 50 μl of 3 M sodium acetate (pH 5.2). Transfer the DNA solution to a microfuge tube and recover the DNA by precipitation with 2 volumes of ethanol for 10 minutes at 4°C and centrifugation at >10,000*g* for 15 minutes at 4°C.

CHROMATOGRAPHY THROUGH BIO-GEL A-150m OR SEPHAROSE CL-4B

This is a modification of an unpublished procedure developed by F. DeNoto and H. Goodman.

1. Prepare the plasmid DNA as described in steps 1–4, page 1.51.

2. Extract the solution once with an equal volume of phenol equilibrated in TE (pH 8.0) (see Appendix B).

3. Layer up to 1 ml of the aqueous phase on a column of Bio-Gel A-150m or Sepharose CL-4B (1 cm × 10 cm) equilibrated in TE (pH 8.0) and 0.1% SDS.

   For instructions on the preparation of gel-filtration columns, see Chapter 8, page 8.70.

4. Wash the DNA into the column, apply a reservoir of TE (pH 8.0) containing 0.1% SDS, and immediately begin to collect 0.5-ml fractions.

5. When 15 fractions have been collected, clamp off the bottom of the column. Analyze 10 μl of each fraction by electrophoresis through a 0.7% agarose gel or by ethidium bromide fluorescence (see Appendix E) in order to locate the plasmid DNA.

6. Pool the fractions that contain plasmid DNA. Recover the DNA by precipitation with 2 volumes of ethanol for 10 minutes at 4°C and centrifugation at $>10{,}000g$ for 15 minutes at 4°C.

Note

It is very difficult to remove all traces of plasmid DNA from the column of Bio-Gel A-150m or Sepharose CL-4B. To eliminate the possibility of contamination, use each column only once.

Strategies for Cloning in Plasmid Vectors

In principle, cloning in plasmid vectors is very straightforward. The plasmid DNA is cleaved with a restriction enzyme and joined in vitro to foreign DNA. The resulting recombinant plasmids are then used to transform bacteria In practice, however, the plasmid vector must be carefully chosen to minimize the effort required to identify and characterize recombinants The major difficulty is distinguishing between plasmids that contain inserted foreign DNA and vector molecules that have recircularized without insertion of foreign DNA Recircularization of the vector can be limited to some extent by adjusting the concentrations of the foreign DNA and vector DNA in the ligation reaction However, a number of procedures, which are described below, have been developed either to reduce recircularization of the vector still further or to distinguish recombinants from nonrecombinants by genetic techniques

Because of their reliability and ease of handling, plasmids have become the workhorses of molecular cloning They simply work better than any other vectors when the DNA to be cloned is of simple structure and small size (<10 kb) However, there is one type of DNA that is difficult to propagate in plasmids—DNA that contains long, head-to-head palindromic sequences (Collins and Hohn 1978, Lilley 1980, Collins 1981; Mizuuchi et al. 1982; Hagan and Warren 1983) Such sequences are lethal to their carrier plasmid, perhaps because they inhibit DNA replication by interfering with the passage of replication forks or because they deleteriously affect the state of supercoiling of the plasmid Plasmids carrying palindromic sequences are therefore prone to suffer deletions that destroy the symmetry.

STRATEGIES FOR LIGATION

Several strategies are available to ligate fragments of foreign DNA to plasmid vectors The choice among them depends on the nature of the termini of the foreign DNA fragment and the nature of the restriction sites in the plasmid vector and the foreign DNA.

The Nature of the Ends of the Foreign DNA Fragment

Recommendations for cloning foreign DNAs carrying various types of termini are given in Table 1.2

FRAGMENTS CARRYING NONCOMPLEMENTARY PROTRUDING TERMINI

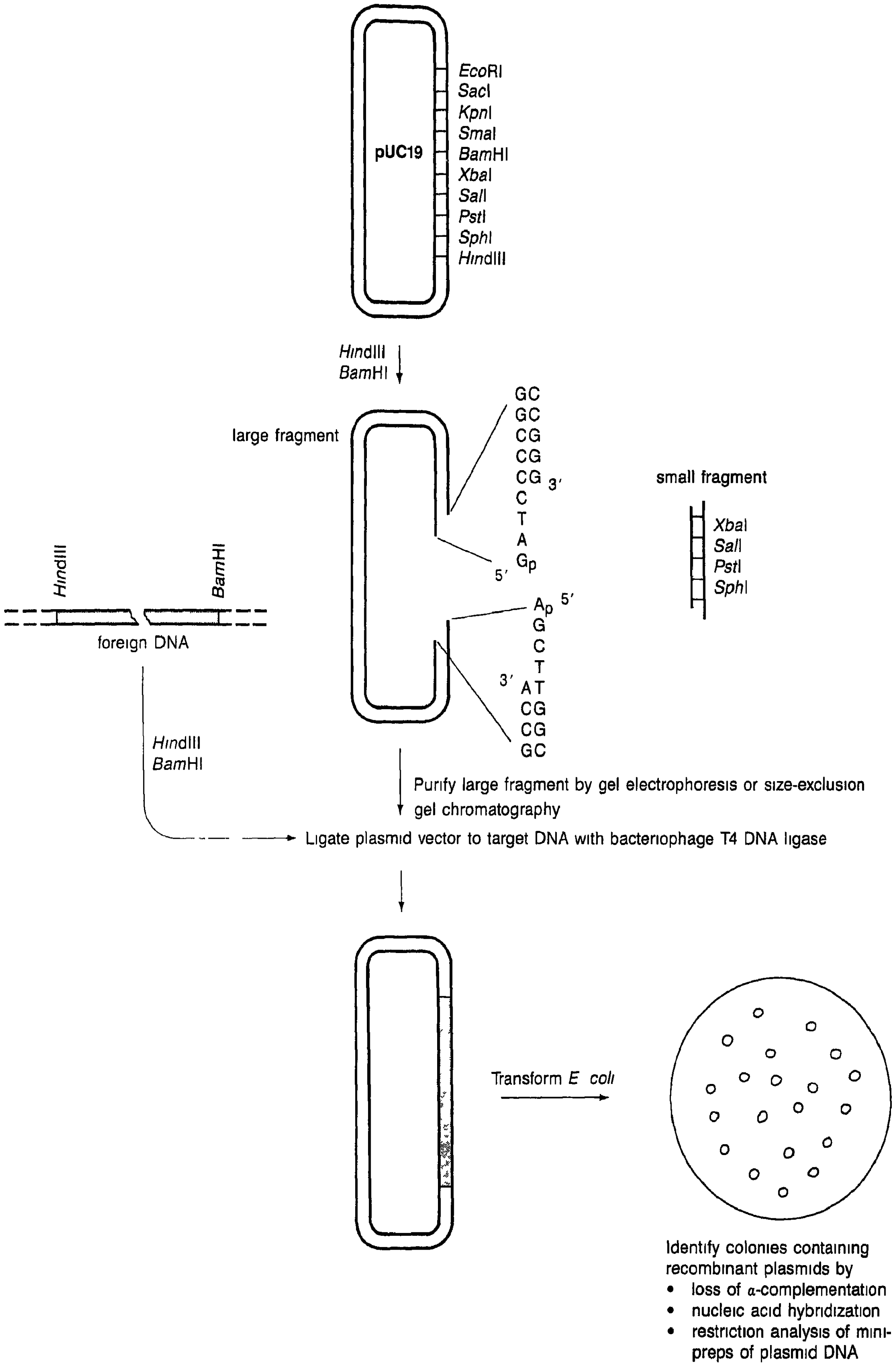
The easiest fragments to clone carry noncomplementary protruding termini generated by digestion with two different restriction enzymes Most plasmid vectors in common use contain polycloning sites that consist of recognition sequences for several different restriction enzymes. Given the large variety of polycloning sites currently available, it is almost always possible to find a vector carrying restriction sites that are compatible with the termini of the

TABLE 1.2 Ligation of Fragments of Foreign DNA to Plasmid Vectors

Termini carried by fragment of foreign DNA	**Requirements for cloning**	**Comments**
Blunt-ended	high concentrations of DNAs and ligase	background of nonrecombinant clones can be high restriction sites at junctions between plasmid and foreign DNAs may be eliminated recombinant plasmids may carry tandem copies of foreign DNA
Different protruding termini	for maximum efficiency requires purification of plasmid vector after digestion with two restriction enzymes	restriction sites at junctions between plasmid and foreign DNAs are usually conserved background of nonrecombinant clones is low foreign DNA is inserted in only one orientation within recombinant plasmid
Identical protruding termini	phosphatase treatment of linear plasmid DNA	restriction sites at junctions between plasmid and foreign DNAs are usually conserved foreign DNA can be inserted in either orientation recombinant plasmids may carry tandem copies of foreign DNA

fragment of foreign DNA. The fragment of foreign DNA is then inserted into the vector by a process known as directional cloning (Figure 1 6) For example, the vector pUC19 can be digested with *Bam*HI and *Hin*dIII and, after digestion, the large fragment of the vector can be purified from the small remnant of the polycloning site by gel electrophoresis or size-exclusion gel chromatography This vector can then be ligated to a segment of foreign DNA that contains cohesive termini compatible with those generated by *Bam*HI and *Hin*dIII The resulting circular recombinant is then used to transform *E coli* to ampicillin resistance Because of the lack of complementarity between the *Hin*dIII and *Bam*HI protruding ends, the vector fragment cannot circularize efficiently and transforms *E coli* very poorly Therefore, most of the bacterial cells resistant to ampicillin contain plasmids that carry a foreign DNA segment that forms a bridge between the *Hin*dIII and *Bam*HI sites Of course, different combinations of enzymes can be used depending on the particular segment of foreign DNA

When preparing vectors for directional cloning, try to avoid using restriction sites that are directly adjacent to one another in the polycloning site. After one of these sites has been cleaved, the second site will be located within a few base pairs of one end of the linear DNA molecule. This is too close to allow efficient cleavage by many restriction enzymes. It is therefore important to check that both restriction enzymes have digested the vector to completion. This can be done in two ways:



Purify large fragment by gel electrophoresis or size-exclusion gel chromatography

Ligate plasmid vector to target DNA with bacteriophage T4 DNA ligase

Identify colonies containing recombinant plasmids by
- loss of α-complementation
- nucleic acid hybridization
- restriction analysis of mini-preps of plasmid DNA

FIGURE 1.6
Directional cloning in plasmid vectors.

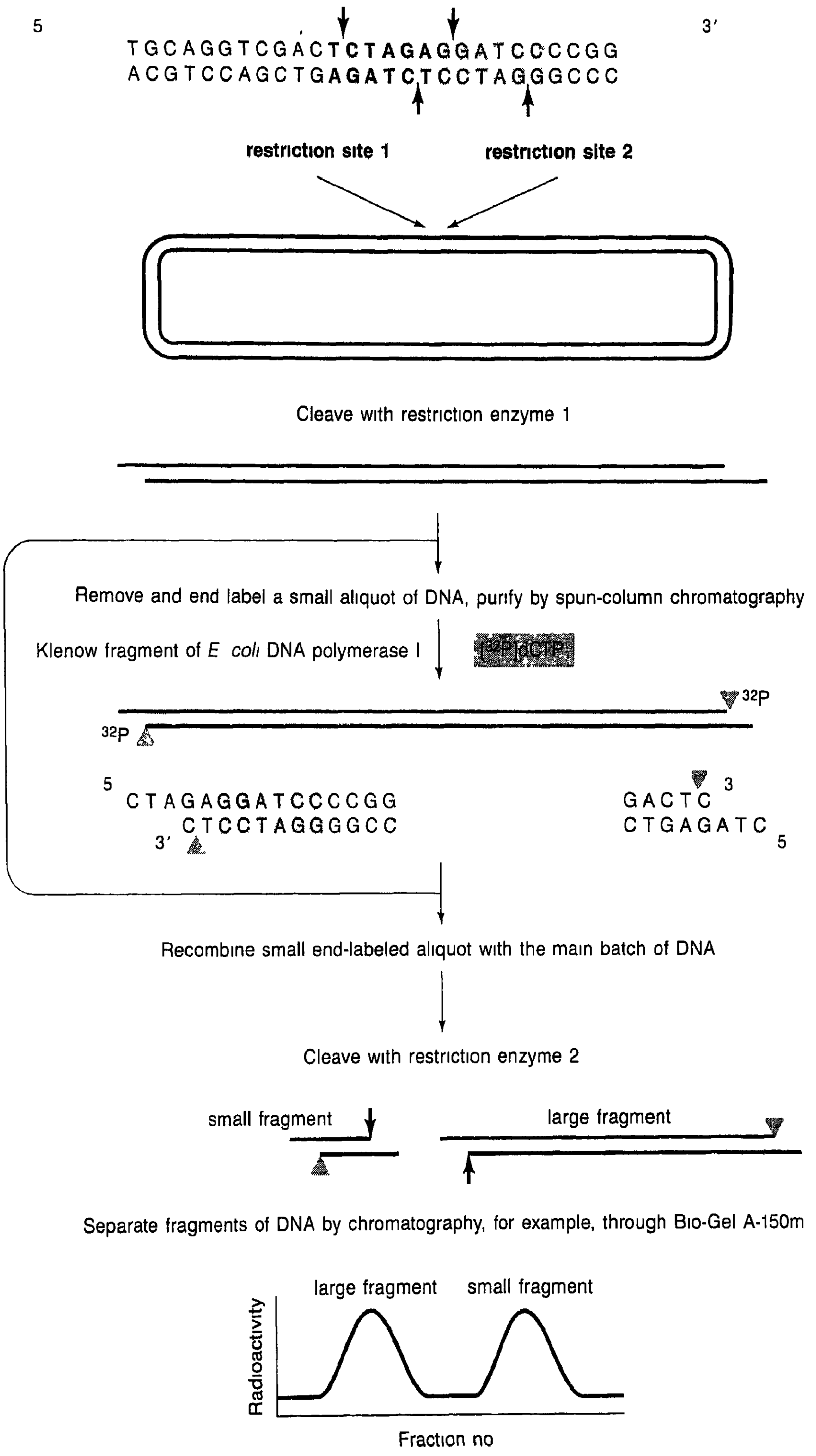
- Digest the vector with one of the two restriction enzymes. When using restriction enzymes that work optimally in buffers of different ionic strengths, use the enzyme that prefers the lower concentration of salt At the end of the digestion, check an aliquot of the DNA by gel electrophoresis If all of the plasmid DNA has been converted from circular to linear molecules, adjust the salt concentration appropriately and add the second enzyme At the same time, set up a separate pilot reaction that contains circular plasmid DNA and only the second of the two restriction enzymes When all of the DNA in the pilot reaction has been converted to a linear form (as determined by gel electrophoresis), purify the large segment of plasmid DNA by gel electrophoresis or size-exclusion gel chromatography
- A more stringent way to check that digestion is complete is shown in Figure 1 7. An aliquot of DNA that has been cleaved with the first restriction enzyme is end-labeled, purified by spun-column chromatography, mixed with unlabeled linear DNA, and then digested with the second enzyme Complete digestion should result in the release of a small fragment of DNA that carries 50% of the radioactivity This can be detected by chromatography or by gel electrophoresis and autoradiography

FRAGMENTS CARRYING IDENTICAL TERMINI (BLUNT-ENDED OR PROTRUDING)

Fragments of foreign DNA carrying identical termini (either blunt-ended or protruding) must be cloned in a linearized plasmid vector bearing compatible ends During the ligation reaction, the foreign DNA and the plasmid have the capacity to circularize and to form tandem oligomers It is therefore necessary to carefully adjust the concentrations of the two types of DNA in the ligation reaction to optimize the number of "correct" ligation products (see page 1 63) In addition, removal of the 5′-phosphate groups with alkaline phosphatase is frequently used to suppress self-ligation and circularization of the plasmid DNA. During ligation in vitro, bacteriophage T4 DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5′-phosphate group and the other contains a 3′-hydroxyl group Recircularization of plasmid DNA can therefore be minimized by removing the 5′-phosphates from both termini of the linear DNA with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) (Seeburg et al 1977; Ullrich et al 1977) However, a foreign DNA segment with 5′-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA to give an open circular molecule containing two nicks (Figure 1.8) Because circular DNA (even nicked circular DNA) transforms much more efficiently than linear plasmid DNA, most of the transformants will contain recombinant plasmids A protocol for phosphatase treatment of plasmid DNA is given on pages 1.60–1.61

FRAGMENTS CARRYING BLUNT ENDS

An additional complication arises when the fragment of foreign DNA carries blunt ends. Such DNAs are ligated much less efficiently than DNAs carrying protruding complementary termini. Ligation reactions involving blunt-



Cleave with restriction enzyme 1

Remove and end label a small aliquot of DNA, purify by spun-column chromatography

Recombine small end-labeled aliquot with the main batch of DNA

Cleave with restriction enzyme 2

Separate fragments of DNA by chromatography, for example, through Bio-Gel A-150m

FIGURE 1.7
Digestion of plasmid DNA with two restriction enzymes See text for details.

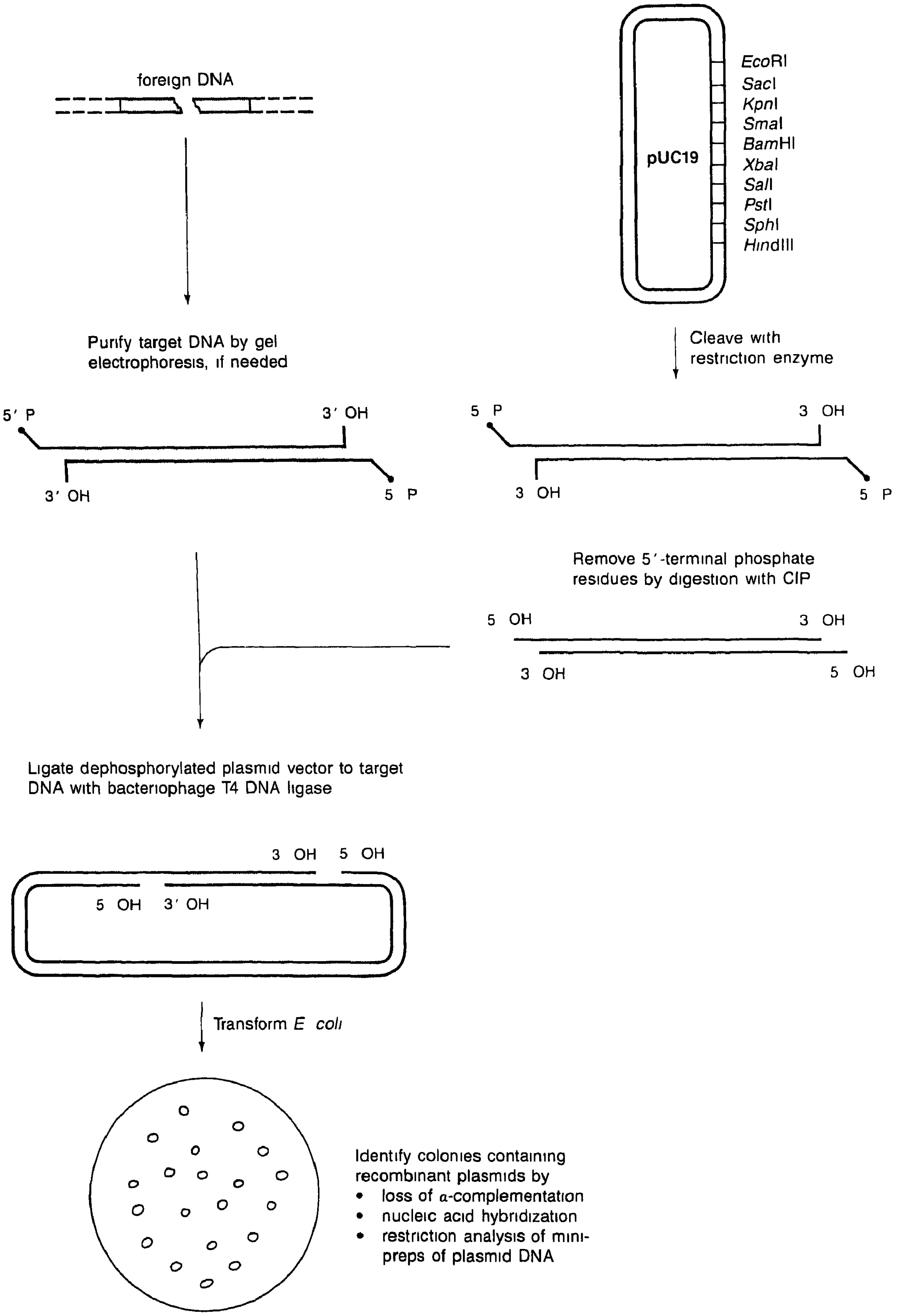

foreign DNA
pUC19
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
Purify target DNA by gel electrophoresis, if needed
Cleave with restriction enzyme
5′ P
3′ OH
3′ OH
5 P
5 P
3 OH
3 OH
5 P
Remove 5′-terminal phosphate residues by digestion with CIP
5 OH
3 OH
3 OH
5 OH
Ligate dephosphorylated plasmid vector to target DNA with bacteriophage T4 DNA ligase
3 OH
5 OH
5 OH
3′ OH
Transform E coli
Identify colonies containing recombinant plasmids by
• loss of α-complementation
• nucleic acid hybridization
• restriction analysis of minipreps of plasmid DNA

FIGURE 1.8
Use of phosphatase to prevent recircularization of vector DNA

ended molecules therefore require much higher concentrations both of bacteriophage T4 DNA ligase and of foreign and plasmid DNAs. In addition, the efficiency of this type of reaction is often increased by addition of low concentrations of substances such as polyethylene glycol (see page 1.70).

The Nature of the Restriction Sites in the Plasmid Vector and the Foreign DNA

The variety of restriction sites in plasmid vectors is now extremely large, and it is often possible to find a vector that carries exactly the same restriction sites as the fragment of foreign DNA itself This has the advantage of allowing the foreign DNA to be recovered from the recombinant plasmid by digestion with the appropriate restriction enzymes. Alternatively, the fragment can be inserted into the vector at any sites that generate compatible termini For example, the restriction enzymes *Bam*HI and *Bgl*II, which recognize different hexanucleotide sequences, generate restriction fragments with identical protruding termini Thus, fragments of foreign DNA prepared by digestion with *Bgl*II can be cloned into a plasmid digested with *Bam*HI This usually generates junction sequences that cannot be cleaved by the restriction enzyme used to prepare either the foreign DNA or the vector In many cases, however, the fragment can be released from the recombinant plasmid by digestion with restriction enzymes that cleave in flanking polycloning sequences.

Occasionally, it is impossible to find a suitable match between restriction sites in the plasmid and those at the ends of the foreign DNA There are two solutions to this problem.

- Synthetic linkers or adapters can be ligated to the termini of the linearized plasmid and/or fragment of foreign DNA (see Appendix F).
- DNA fragments with recessed 3′ termini can be partially filled in controlled reactions using the Klenow fragment of *E. coli* DNA polymerase I. As discussed in Chapter 9, this procedure often generates complementary termini from restriction sites that are otherwise incompatible, thus facilitating ligation of the vector and foreign DNAs Because partial filling eliminates the ability of termini on the same molecule to pair with one another, the frequencies of circularization and self-oligomerization during the ligation reaction are also reduced (Hung and Wensink 1984; Zabarovsky and Allikmets 1986).

DEPHOSPHORYLATION OF LINEARIZED PLASMID DNA

Calf intestinal alkaline phosphatase (CIP), a dimeric glycoprotein, is the enzyme of choice to remove 5'-phosphate groups from linear DNAs Because each monomer of CIP carries two atoms of zinc that are essential for the activity of the enzyme (Simsek et al 1973), the buffer used for dephosphorylation should contain $ZnCl_2$ at a final concentration of 1 mM At the end of the dephosphorylation reaction, CIP is removed by digestion with a small amount of proteinase K in the presence of EDTA, and the dephosphorylated DNA is then purified by extraction with phenol chloroform

1. Digest closed circular plasmid DNA (10–20 μg) with a two- to threefold excess of the desired restriction enzyme for 1 hour Remove an aliquot (0.3 μg), and analyze the extent of digestion by electrophoresis through a 0 8% agarose gel, using undigested plasmid DNA as a marker If digestion is incomplete, add more restriction enzyme and continue the incubation

2. When digestion is complete, extract the sample with phenol chloroform and precipitate the DNA with 2 volumes of ethanol for 15 minutes at 0°C Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge, and redissolve it in 90 μl of 10 mM Tris Cl (pH 8 3) Remove an aliquot of the DNA (200 ng) and store it at −20°C

3. To the remainder of the DNA, add 10 μl of 10× CIP dephosphorylation buffer and the appropriate amount of CIP and incubate under the appropriate conditions

10× CIP dephosphorylation buffer

10 mM $ZnCl_2$
10 mM $MgCl_2$
100 mM Tris · Cl (pH 8.3)

	Protruding 5' termini	**Blunt or recessed termini**
Amount of CIP required	1 unit/100 pmoles	1 unit/2 pmoles
Incubation conditions	30 minutes at 37°C	15 minutes at 37°C Then add another aliquot of CIP and continue incubation for a further 45 minutes at 55°C

2 μg of a linearized plasmid DNA 5 kb in length contains approximately 1 4 pmoles of 5'-terminal phosphate residues (see Appendix C)

4. At the end of the incubation period, add SDS and EDTA (pH 8.0) to final concentrations of 0.5% and 5 mM, respectively Mix well, and add proteinase K to a final concentration of 100 μg/ml. Incubate for 30 minutes at 56°C.

Proteinase K is used to digest the CIP, which must be completely removed if the subsequent ligations are to work efficiently An alternative method is to inactivate the CIP by heating to 65°C for 1 hour (or 75°C for 10 minutes) in the presence of 5 mM EDTA (pH 8 0), and then to purify the dephosphorylated DNA by extraction with phenol chloroform

5. Cool the reaction to room temperature, and extract once with phenol and once with phenol.chloroform Add 0 1 volume of 3 M sodium acetate (pH 7 0) Mix well, and add 2 volumes of ethanol. Mix well, and store at 0°C for 15 minutes. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge Wash the pellet with 70% ethanol at 4°C and recentrifuge. Redissolve the precipitated DNA in TE (pH 7 6) at a concentration of 100 μg/ml. Store in aliquots at −20°C

Notes

i The pH of the 3 M sodium acetate solution is 7.0 rather than the usual 5 2 At acid pH, EDTA precipitates from solution if its concentration exceeds 5–10 mM

ii Until recently, CIP was supplied as a suspension in a slurry of ammonium sulfate However, it may now be obtained as a stabilized solution (in 30 mM triethanolamine buffer [pH 7.6], 3 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$) It should be stored undiluted in this buffer (not frozen)

iii. To remove 5′ phosphates from RNA, use 0 01 unit of CIP per picomole of 5′ termini Incubate for 15 minutes at 37°C, followed by 30 minutes at 55°C

Test Ligations and Transformations

The best way to assay whether dephosphorylation has been successful is to set up a series of test ligations and transformations as follows

1. Set up the following test ligations·

   Ligation A. 200 ng of dephosphorylated plasmid DNA

   Ligation B: 200 ng of dephosphorylated plasmid DNA plus 200 ng of a foreign DNA fragment carrying compatible phosphorylated termini

   Ligation C: 200 ng of linearized plasmid DNA that has not been treated with CIP (the aliquot set aside in step 2, page 1 60)

2. To each test ligation, add·

10× ligation buffer	1 μl
10 mM ATP	1 μl
H_2O to 10 μl	

   10× Ligation buffer

   0.5 M Tris · Cl (pH 7.6)
   100 mM $MgCl_2$
   100 mM dithiothreitol
   500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

3. Add 0 5 Weiss unit of bacteriophage T4 DNA ligase to each test ligation Incubate the reactions for 4–8 hours at 16°C (For definition of Weiss unit, see page 1 68)

4. Transform competent *E coli* with 0 0, 0.01, 0 1, and 1 μl of each of the ligation reactions (see page 1 77 or 1 82) As a positive control, transform *E coli* with a standard solution containing a known amount of a closed circular plasmid (e g., 10 pg of pUC18 or pUC19) Plate 1, 10, and 50 μl of each of the transformation reactions on plates containing the appropriate antibiotic After incubation overnight at 37°C, count the number of colonies on each plate. Dephosphorylation should reduce the transformation efficiency of linear plasmid DNA by a factor of at least 50 (ligation A vs. ligation C). Ligation of foreign DNA (ligation B) to dephosphorylated linear plasmid DNA should increase the transformation frequency by a factor of at least 5 (ligation B vs ligation C).

   It is essential to include two controls in *every* transformation experiment
   - A mock transformation reaction that receives no DNA at all
   - A transformation reaction that receives a known amount of a standard preparation of closed circular plasmid DNA

   Most of the time, transformations work well and the controls then appear to be superfluous. However, the controls come into their own on the occasions when things go wrong For example, the presence of many colonies on plates that receive mock-transformed bacteria may indicate that the medium lacks the correct antibiotic A paucity of colonies on plates receiving bacteria transformed with plasmids under construction can only be interpreted if a positive control using a standard DNA has been included

LIGATION REACTIONS

Setting Up Ligation Reactions Between Foreign DNA and Plasmid Vectors

Ligation of a segment of foreign DNA to a linearized plasmid vector involves the formation of new bonds between phosphate residues located at the 5′ termini of double-stranded DNA and adjacent 3′-hydroxyl moieties When both strands of the plasmid vector carry 5′-phosphate residues, four new phosphodiester bonds are generated However, when the plasmid DNA has been dephosphorylated, only two new phosphodiester bonds can be formed. In this case, the resulting hybrid molecules carry two single-strand nicks (see Figure 1 8) that are repaired after the hybrids have been introduced into competent bacteria.

The formation of phosphodiester bonds between adjacent 5′-phosphate and 3′-hydroxyl residues can be catalyzed in vitro by two different DNA ligases—*E coli* DNA ligase and bacteriophage T4 DNA ligase. For virtually all cloning purposes, bacteriophage T4 DNA ligase is the enzyme of choice because it will join blunt-ended DNA fragments efficiently under normal reaction conditions.

Ligation of one end of DNA to another can be regarded as a bimolecular reaction whose velocity under standard conditions is determined solely by the concentration of compatible DNA termini This is true no matter whether the termini are located on the same molecule of DNA (intramolecular ligation) or on different molecules (intermolecular ligation). Consider the simple case of a ligation mixture that contains only one species of DNA—a phosphorylated vector DNA prepared by cleavage of a plasmid with a single restriction enzyme that generates cohesive termini In the first and rate-limiting stage of the reaction, two of these termini must pair to form a substrate on which the ligase can subsequently act If the concentration of DNA in the reaction is low, the chances are good that these two termini will belong to the same molecule of DNA (because a terminus on one end of a DNA molecule has a higher probability of finding the terminus on the other end than of finding a terminus on a different DNA molecule). Thus, at low concentrations of DNA, recircularization of the plasmid DNA will occur with high efficiency If the concentration of DNA in the ligation reaction is increased, a given end of DNA is more likely to encounter a terminus located on another molecule before intramolecular ligation occurs. At high DNA concentrations, therefore, the initial products of ligation will be dimers and larger oligomers of the plasmid

A theoretical discussion of the effects of DNA concentration on the nature of ligation products is presented by Dugaiczyk et al. (1975; see also *Bethesda Res Lab Focus* vol 2, nos. 2 and 3) In brief, the ratio of cyclized to concatemeric ligation products depends on two parameters j and i. j is the effective concentration of one end of a DNA molecule in the neighborhood of the other end of the same molecule The value given to j is based on the assumption that DNA in solution behaves as a random coil. Thus, j is inversely proportional to the length of the DNA molecule (as the DNA gets larger, the ends are less likely to interact). j is therefore a constant for a DNA molecule of a given length and is independent of DNA concentration:

$$j = \left(\frac{3}{2\pi lb}\right)^{3/2}$$

where l is the length of DNA in centimeters and b is the length of a randomly coiled segment of DNA. The value of b is dependent on the ionic strength of the buffer, which affects the rigidity of the DNA.

i is a measure of all complementary termini in the solution. For double-stranded DNA with self-complementary cohesive termini,

$$i = 2\ N_0 M \times 10^{-3}\ \text{ends/ml}$$

where N_0 is Avogadro's number and M is the molar concentration of the DNA.

Theoretically, when $j = i$, the end of a given DNA molecule is equally likely to make contact either with the other end of the same molecule or the end of a different molecule. Thus, under these conditions, both circles and concatemers should be generated at equal rates during the initial stages of the reaction. When $j > i$, recircularization is favored, and when $i > j$, concatemers are favored. Figure 1.9 shows the relationship between the size of a segment of DNA and the concentration required in a ligation mixture to achieve $j{:}i$ ratios of 0.5, 1, 2, and 5 (Dugaiczyk et al. 1975).

Consider now a ligation mixture that contains, in addition to the linear plasmid, a fragment of foreign DNA with compatible termini. The efficiency with which a given ligation mixture produces monomeric circular recombinant genomes is affected not only by the absolute concentration of termini in the reaction, but also by the relative concentrations of the termini of the plasmid and foreign DNAs. The maximum yield of useful recombinants is obtained when i is two- to threefold greater than j (i.e., when the absolute concentration of termini is high enough to favor intermolecular ligation but not so high as to cause extensive formation of oligomeric molecules) and when the concentration of the termini of foreign DNA is approximately twice the concentration of the termini of the plasmid DNA. Under these circumstances, approximately 40% of the final ligation products will consist of monomeric plasmid:foreign DNA chimeras. Figure 1.10 shows the theoretical yield of such chimeras at the end of a ligation reaction when the ligation mixture contains a constant amount of linearized plasmid ($j{:}i = 3$) and increasing amounts of foreign DNA carrying compatible termini. Figure 1.11 shows the theoretical yields of useful recombinants when other types of ligation reactions are carried out: (1) when the linearized plasmid DNA has been dephosphorylated; (2) when the vector has been prepared by cleavage of plasmid DNA with two different restriction enzymes but not further purified (This ligation reaction contains three different DNA species—the plasmid vector, the foreign DNA fragment, and the stuffer fragment of the plasmid.), and (3) when the plasmid has been cleaved with two different restriction enzymes and then purified from the stuffer fragment.

As the concentration of foreign DNA increases, these curves rise steeply, pass through a maximum, and then decrease slowly. In theory, these curves can be used to predict the exact amounts of vector and foreign DNAs that will yield the maximum amount of useful ligation products. In practice, however, it is often not possible to know the exact concentration of each DNA species in a ligation reaction. Often, these DNAs have been purified by gel elec-

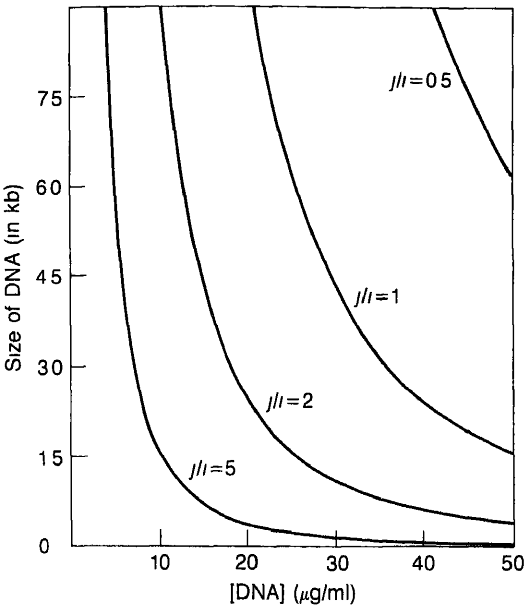


FIGURE 1.9

The relationship between the size of DNA and its concentration at four different $j:i$ ratios. The figure (modified from Dugaiczyk et al. 1975) shows graphs of the equation

$$\text{m.w.} = \left(\frac{51.1}{j/i\,[\text{DNA}]}\right)^2$$

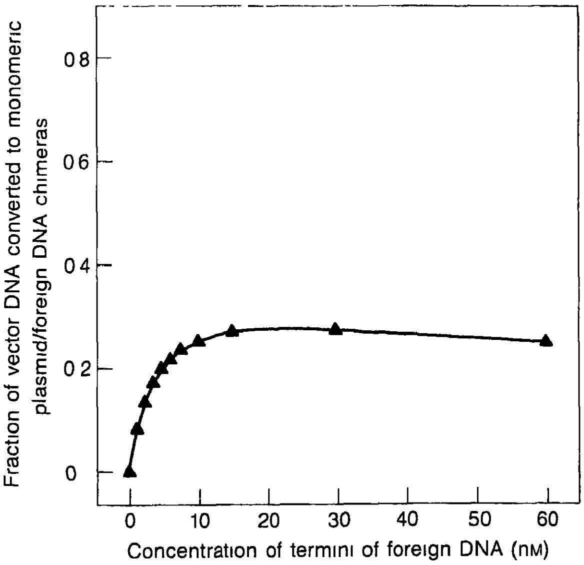


FIGURE 1.10

The efficiency with which monomeric circular chimeras are generated when a fixed amount of linearized phosphorylated plasmid DNA with cohesive termini is ligated to increasing quantities of a segment of foreign DNA that carries compatible termini. This figure was provided by C. Brewer (University of Texas Southwestern Medical Center).

trophoresis and are present in such small quantities that only rough estimates of their concentrations are available. Furthermore, it is likely that some of the DNA termini have been damaged during preparation. On those rare occasions when it is essential to obtain the maximum yield of useful recombinants (e.g., when constructing a cDNA library), it is therefore advisable to carry out a series of pilot reactions to determine the optimal concentrations and ratio of vector DNA:foreign DNA. For most routine cloning purposes, this is unnecessary. The curves in Figures 1.9, 1.10, and 1.11 show that reasonable numbers of useful recombinants will be obtained when the ratio of plasmid DNA:foreign DNA ≤ 1.

In summary, ligation reactions involving linearized phosphorylated plasmid DNA with cohesive termini should contain

- Sufficient vector DNA to achieve a j:i ratio of >1 and <3. For a plasmid the size of pUC18, this means that the ligation reaction should contain between 20 μg and 60 μg of vector DNA per milliliter.
- An equal or slightly greater concentration of termini of foreign DNA than of vector DNA. If the concentration of foreign DNA is substantially lower than that of the vector, the number of useful ligation products will be very low (see Figures 1.10 and 1.11) and it may be difficult to identify the small

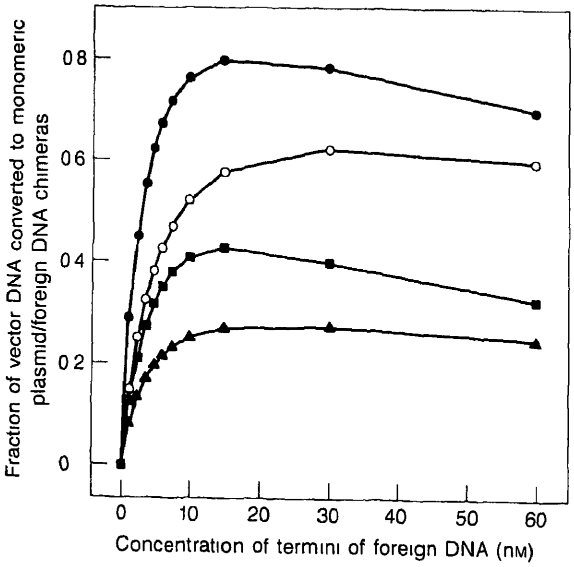


FIGURE 1.11

The efficiency with which monomeric circular chimeras are generated when a fixed amount of vector DNA is ligated to increasing quantities of a segment of foreign DNA that carries compatible termini.

(●) Vector cleaved with two different restriction enzymes, stuffer fragment removed by gel electrophoresis. (○) Vector cleaved with two different restriction enzymes; stuffer fragment not removed. (■) Vector cleaved with one restriction enzyme and dephosphorylated. (▲) Vector cleaved with one restriction enzyme and not dephosphorylated.

This figure was provided by C. Brewer (University of Texas Southwestern Medical Center).

proportion of transformed bacterial colonies that carry recombinant plasmids. In this case, it is advisable to consider taking steps to reduce the background of colonies carrying nonrecombinant plasmids either by treating the linearized plasmid DNA with phosphatase or by adopting another cloning strategy so that the recombinant plasmid can be constructed by directional cloning.

Ligation of Cohesive Termini

1. Digest the plasmid and foreign DNAs with the appropriate restriction enzymes. If necessary, isolate the desired fragments by gel electrophoresis and/or treat the plasmid DNA with phosphatase. Purify the DNAs by extraction with phenol:chloroform and ethanol precipitation. Redissolve the DNAs in TE (pH 7.6) at a concentration of 100 μg/ml.

2. Set up the ligation mixtures as follows:

   a. Transfer 0.1 μg of the vector DNA to a sterile microfuge tube. Add an equimolar amount of foreign DNA.

   b. Add H_2O to 7.5 μl, and warm the solution to 45°C for 5 minutes to melt any cohesive termini that have reannealed. Chill the mixture to 0°C.

   c. Add

10× bacteriophage T4 DNA ligase buffer	1 μl
bacteriophage T4 DNA ligase	0.1 Weiss unit
5 mM ATP	1 μl

Incubate the reactions for 1–4 hours at 16°C.

10× Bacteriophage T4 DNA ligase buffer

200 mM Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C.

Set up two additional control reactions that contain (1) the plasmid vector alone and (2) the fragment of foreign DNA.

If insufficient foreign DNA is available, use 50–100 ng of the plasmid DNA per ligation and add as much foreign DNA as possible without increasing the volume of the ligation reaction to greater than 10 μl.

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit as determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units as defined by New England Biolabs. 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

Bacteriophage T4 DNA ligase is supplied in concentrated solution (1–5 units/μl). This should be diluted and stored at a concentration of 100 units/ml in 20 mM Tris·Cl (pH 7.6), 60 mM KCl, 5 mM dithiothreitol, 500 μg/ml bovine serum albumin, 50% glycerol. Bacteriophage T4 DNA ligase is stable at this concentration in this buffer for 3 months at −20°C.

3. Use 1–2 μl of each of the ligation reactions to transform competent *E coli* as described on pages 1.77–1 81 or 1.82–1.84.

Ligation of Blunt-ended DNA

Bacteriophage T4 DNA ligase, unlike *E. coli* DNA ligase, can catalyze the ligation of blunt-ended fragments of DNA (Sgaramella and Khorana 1972, Sgaramella and Ehrlich 1978). Since DNA can easily be rendered blunt-ended, this is an extremely useful property because it allows any DNA molecule to be ligated to any other However, ligation of blunt-ended termini is a comparatively inefficient reaction and requires four conditions

- Low concentrations (0 5 mM) of ATP (Ferretti and Sgaramella 1981)
- The absence of polyamines such as spermidine
- Very high concentrations of ligase (50 Weiss units/ml)
- High concentrations of blunt-ended termini

CONDENSING AGENTS

The problem of attaining adequate concentrations of blunt-ended DNA can be avoided by including in the reaction mixture substances that increase macromolecular crowding and cause DNA molecules to condense into aggregates, for example, polyethylene glycol (Pheiffer and Zimmerman 1983, Zimmerman and Pheiffer 1983, Zimmerman and Harrison 1985) or hexamminecobalt chloride (Rusche and Howard-Flanders 1985) These substances have two effects on ligation reactions.

1. They accelerate the rate of ligation of blunt-ended DNA by 1 to 3 orders of magnitude This increase allows ligation reactions to be formed at low enzyme and DNA concentrations
2. They alter the distribution of ligation products Intramolecular ligation is suppressed, and the ligation products are created exclusively by intermolecular joining events Thus, even at concentrations of DNA that favor circularization ($j:i = 10$), all the DNA products are linear multimers

The following information may be useful when setting up ligation reactions that contain condensing agents

Polyethylene glycol (PEG 8000)

- Stock solutions (40%) of PEG 8000 made in deionized water may be stored frozen in small aliquots, but they should be thawed and warmed to room temperature before being added to the ligation mixture Maximal stimulation is observed in ligation mixtures containing 15% PEG 8000 Prepare reaction mixtures at 0°C by mixing all components except the PEG 8000 and the bacteriophage T4 DNA ligase Then add an appropriate volume of PEG 8000 (at room temperature) and mix Add the enzyme and incubate the reaction at 20°C.
- Maximum stimulation of ligation is observed when the ligation mixture contains 0.5 mM ATP and 5 mM $MgCl_2$. Even a modest increase in the

concentration of ATP or decrease in the concentration of $MgCl_2$ greatly reduces the degree of stimulation (Pheiffer and Zimmerman 1983).

- PEG 8000 at a concentration of 15% stimulates ligation of DNA molecules carrying cohesive termini by a factor of 10–100. The major products of the reaction are tandem concatemers.
- PEG 8000 stimulates blunt-end ligation of synthetic oligomers as short as 8 nucleotides in length. In this respect, it differs from hexamminecobalt chloride

Hexamminecobalt chloride

- Hexamminecobalt chloride is stored as a 10 mM stock solution in water at −20°C Stimulation of ligation by hexamminecobalt chloride is highly concentration-dependent and is maximal in ligation mixtures containing the salt at a concentration of 1.0–1 5 μM Hexamminecobalt chloride increases the efficiency of blunt-end ligation about 50-fold, whereas ligation of cohesive termini is increased only 5-fold (Rusche and Howard-Flanders 1985)
- In the presence of monovalent cations (30 mM KCl), some stimulation of blunt-end ligation still occurs, but the distribution of ligation products changes Instead of ligation products that are derived exclusively from intermolecular reactions, circular DNA molecules now predominate.
- Unlike PEG 8000, hexamminecobalt chloride does not significantly increase the rate of ligation of synthetic oligonucleotides

Rapid Cloning in Plasmid Vectors

The slowest step in cloning in plasmids is the electrophoretic purification of the desired restriction fragment of foreign DNA and the appropriate segment of plasmid DNA. In the protocol given below (adapted from Struhl 1985 by S Michaelis, pers comm.), ligation of plasmid and foreign DNAs is carried out directly in the melted slabs of agarose recovered from the gel used for purification. The method works for both blunt-end ligation and ligation of cohesive termini, although it requires a large amount of ligase and its efficiency is about an order of magnitude lower than the standard procedure

1. Digest the foreign DNA with the appropriate restriction enzyme(s) The amount of foreign DNA digested should be sufficient to yield approximately 0 2 μg of the target fragment The digestion should be carried out in a volume of 20 μl or less In a separate tube, digest 0 5 μg of the vector DNA with the appropriate restriction enzyme(s) in a total reaction volume of 20 μl or less

   If the vector DNA carries identical cohesive termini, it should be treated with phosphatase as follows When digestion with the restriction enzyme(s) is complete, add 2 5 μl of 100 mM Tris Cl (pH 8 3), 10 mM $ZnCl_2$ Add 0 25 unit of calf intestinal alkaline phosphatase and incubate for 30 minutes at 37°C

2. Separate the desired fragments by electrophoresis on an agarose gel The gel must be cast with low-melting-temperature agarose and it must be poured and run in 1× TAE electrophoresis buffer (see Chapter 6, page 6 7) containing ethidium bromide (0 5 μg/ml) rather than the conventional 0 5× TBE

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described on pages 1 49–1 50

3. Examine the gel by long-wavelength ultraviolet illumination From the relative fluorescent intensities of the desired bands, estimate the amounts of DNA that they contain (see Appendix E) Using a razor blade, cut out the desired bands in the smallest possible volume of agarose (usually 40–50 μl) Place the excised slices of gel in separate, labeled microfuge tubes

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

4. Heat the tubes to 70°C for 10–15 minutes to melt the agarose

5. Combine aliquots of the melted gel slices in a fresh tube prewarmed to 37°C The final volume of the combined aliquots should be 10 μl or less, and the molar ratio of foreign DNA:plasmid vector should be approximately 2:1.

   In separate tubes, set up two additional ligations as controls, one containing the plasmid vector alone and the other containing only the fragment of foreign DNA.

6. Incubate the three tubes for 5–10 minutes at 37°C, and then add to each tube 10 μl of ice-cold 2× bacteriophage T4 DNA ligase mixture. Mix the contents of the tubes well before the agarose hardens. Incubate the reactions for 12–16 hours at 16°C.

   2× Bacteriophage T4 DNA ligase mixture is prepared as follows:

1 M Tris·Cl (pH 7.6)	1.0 μl
100 mM $MgCl_2$	1.0 μl
200 mM dithiothreitol	1.0 μl
10 mM ATP	1.0 μl
H_2O	5.5 μl
bacteriophage T4 DNA ligase	1 Weiss unit

   Mix the components in a tube stored in an ice bath.

   For definition of Weiss unit, see page 1.68.

7. Towards the end of the ligation, remove from storage at −70°C three tubes containing 200 μl of frozen competent *E. coli* each (see page 1.80). As soon as the cells have thawed, place them in an ice bath. Immediately proceed to step 8.

8. Remelt the agarose in the ligation mixtures by heating them to 70°C for 10–15 minutes.

9. Immediately add 5 μl of one of the ligation mixtures to 200 μl of competent *E. coli*. Mix the contents of the tube quickly by gentle shaking. Repeat this procedure with 5 μl taken from each of the remaining ligation mixtures. Store the transformation mixtures on ice for 30 minutes.

10. Proceed with the remainder of the transformation protocol (step 13, page 1.80).

Preparation and Transformation of Competent E. coli

Most methods for bacterial transformation are based on the observations of Mandel and Higa (1970), who showed that bacteria treated with ice-cold solutions of $CaCl_2$ and then briefly heated could be transfected with bacteriophage λ DNA. The same method was subsequently used to transform bacteria with plasmid DNA (Cohen et al. 1972) and *E. coli* chromosomal DNA (Oishi and Cosloy 1972). Apparently, the treatment induces a transient state of "competence" in the recipient bacteria, during which they are able to take up DNAs derived from a variety of sources. Many variations of this basic technique have since been described, all directed toward optimizing the efficiency of transformation of different bacterial strains by plasmids. Bacteria treated according to the original protocol of Mandel and Higa yield 10^5–10^6 transformed colonies/μg of supercoiled plasmid DNA. This efficiency can be increased 100- to 1000-fold by exposing improved strains of *E. coli* (Kushner 1978; Norgard et al. 1978; Hanahan 1983) to combinations of divalent cations (Kushner 1978) for longer periods of time (Dagert and Ehrlich 1979) and treating the bacteria with DMSO (Kushner 1978), reducing agents, and hexamminecobalt chloride (Hanahan 1983). How these agents act is unknown, as is the mechanism by which plasmid DNA enters competent *E. coli*. These improvements in transformation frequency have occurred solely as a consequence of empirical experimentation. Nevertheless, because of these improvements, the efficiency of transformation has not been a limiting factor in molecular cloning since the late 1970s.

Today, there are two ways to obtain stocks of competent *E. coli*. The first option is to purchase frozen competent bacteria from a commercial source. These products are very reliable and generally yield transformants at frequencies $\geq 10^8$ colonies/μg of supercoiled plasmid DNA. However, they are many times more expensive than competent cells prepared in the laboratory. We therefore recommend that commercially produced competent cells be used only as a yardstick against which to measure the efficiency of transformation of locally generated stocks of competent cells or by investigators who carry out transformations so infrequently that it is not economical for them to expend the effort required to produce their own competent cultures.

The second option is to prepare stocks of competent bacteria locally. The first method given below (Protocol I) works well with strains of *E. coli* such as DH1, DH5, and MM294 and yields competent cultures that can be either used immediately or stored in small aliquots at −70°C until required. If prepared carefully, these competent bacteria can yield up to 10^9 transformed colonies/μg of supercoiled plasmid DNA. However, such high frequencies are required only rarely, for example, when constructing plasmid libraries of cDNAs synthesized from extremely small amounts of mRNA isolated from relatively inaccessible sources. In most other cases, it is possible to use competent bacteria, prepared by a simpler method (Protocol II), that yield approximately 10^7 transformed colonies/μg of supercoiled plasmid DNA.

Transformation of E. coli by High-voltage Electroporation (Electrotransformation)

The method of electroporation was developed originally to introduce DNA into eukaryotic cells (Neumann et al 1982, Potter et al. 1984, Fromm et al 1985, Chu et al. 1987), but it has recently been used to transform *E. coli* (Dower et al 1988; Taketo 1988) and other bacteria (Chassy and Flickinger 1987; Harlander 1987; Somkuti and Steinberg 1987; Fiedler and Wirth 1988; Miller et al. 1988, Powell et al. 1988). Transformation efficiencies of 10^9–10^{10} transformants/μg of DNA have been achieved by optimizing various parameters, including the strength of the electrical field, the length of the electrical pulse, and the concentration of DNA (Dower et al 1988). Higher transformation efficiencies have been observed at higher voltages or longer pulses, but these were offset by a decrease in cell viability The highest levels of transformation were achieved with a combination of field strength and pulse length that resulted in 50–75% cell death

Limited data are available regarding the transformation efficiencies obtained with different plasmid DNAs and bacterial hosts, but yields up to 10^{10} transformants/μg of DNA have been obtained with *E coli* strain LE392 or MC1061 and the plasmid pUC18 (Dower et al. 1988) In general, the authors claim that the efficiency of electrotransformation is 10 to 20 times higher than that obtained with maximally competent cells prepared by chemical methods The effect of plasmid size has not yet been systematically examined, but supercoiled plasmids as large as 25–130 kb have been reported to transform with similar molar efficiencies The efficiency of transformation with linear bacteriophage λ DNA molecules is only 0.1% of that obtained with small supercoiled plasmid DNA A two- to tenfold variation in transformation efficiency has been observed with transformations of different aliquots of the same batch of cells

The preparation of cells for electroporation is considerably easier than preparing competent cells Bacteria are grown to mid-log phase, chilled, centrifuged, and then washed extensively with low-salt buffer to reduce the ionic strength of the cell suspension It is not necessary to form protoplasts. The cells are then resuspended in 10% glycerol at a concentration of 3×10^{10} cells/ml, frozen in dry ice, and stored at −70°C. Aliquots of the cells can then be thawed and used for transformation for at least six months. Electroporation is carried out at low temperatures (0–4°C), since the efficiency of transformation drops as much as 100-fold when carried out at room temperature. Routinely, the DNA is mixed with cold cell suspension and then transferred to a chilled cuvette.

Because of the relatively small size of bacterial cells, electroporation of *E. coli* requires very high field strengths compared to those required for introducing DNA into eukaryotic cells Optimal conditions for bacterial transformation also require small volumes (20–40 μl), so a special high-voltage mini-electrode and sample holder were designed (Dower et al. 1988) This sample holder is available commercially from Bio-Rad, and detailed protocols for bacterial electroporation and a troubleshooting guide are provided by the manufacturer.

Protocol I: Preparation of Fresh or Frozen Competent *E. coli*

The following procedure, which was developed by Hanahan (1983), can yield competent cultures of *E. coli* strains DH1, DH5, and MM294 that can be transformed at frequencies $\geq 5 \times 10^8$ transformed colonies per microgram of supercoiled plasmid DNA The maximum frequency of transformation that can be obtained routinely with most other strains of *E coli* is approximately five- to tenfold lower. However, this is more than adequate for virtually all cloning purposes A few strains of *E coli* (e g , MC1061) are refractory to the method

Three factors appear to be critical for obtaining consistently high frequencies of transformation·

- *The purity of the reagents used in the transformation buffers* It is important to use the highest quality of reagents that can be obtained and to store them in small aliquots in a dark, cool place
- *The state of growth of the cells* For unknown reasons, the highest frequencies of transformation are obtained with cultures that have been grown directly from a master stock stored in freezing medium at 70°C Cultures that have been passaged continuously in the laboratory or that have been stored at 4°C or at room temperature should not be used
- *The cleanliness of the glassware and plasticware* Because the presence of trace amounts of detergent or other chemicals greatly reduces the efficiency of bacterial transformation, it is best to set aside a batch of glassware that is used for no other purpose than to prepare competent bacteria This glassware should be washed and rinsed by hand, filled with pure water (Milli-Q or equivalent), and sterilized by autoclaving The water should be discarded just before the glassware is used

By working with care, it is almost always possible to obtain cultures of competent bacteria that yield 5×10^7 to 1×10^8 transformed colonies per microgram of supercoiled plasmid DNA However, even the most experienced workers find it impossible to consistently generate cultures of competent bacteria that can be transformed at higher frequencies Because of this unpredictable variability, it is necessary to use a standard preparation of supercoiled plasmid DNA to measure the efficiency of transformation of each new batch of competent cells. Before preparing competent bacteria, it is therefore advisable to prepare a large batch of a diluted supercoiled plasmid DNA and store it at −70°C in many small aliquots. This standard preparation should be used to measure the efficiency of transformation of each new batch of competent cells and to check the efficiency of transformation in every experiment. By including a positive control of this type, it is possible to determine whether the failure to obtain transformed colonies in a given experiment is the fault of the competent bacteria or is due to differences between DNA preparations. Aliquots of the competent bacteria can be stored for several months at −70°C without any significant decrease in efficiency

1. Using a sterile platinum wire, streak *E coli* strain DH1 (or DH5 or MM294) directly from a frozen stock (stored at −70°C in freezing medium; see Appendix A) onto the surface of an SOB agar plate. Incubate the plate for 16 hours at 37°C.

   It is not necessary to thaw the frozen stock of bacteria Sufficient bacteria stick to the platinum wire when it is scratched across the surface of the frozen stock A single tube of frozen stock can therefore be used many times

2. Transfer four or five well-isolated colonies into 1 ml of SOB containing 20 mM $MgSO_4$. The colonies should be 1–2 mm in diameter. Disperse the bacteria by vortexing at moderate speed, and then dilute the culture in 30–100 ml of SOB containing 20 mM $MgSO_4$ in a 1-liter flask.

3. Grow the cells for 2.5–3 0 hours at 37°C. For efficient transformation, it is essential that the number of viable cells should not exceed 10^8 cells/ml To monitor the growth of the culture, determine the OD_{600} every 20–30 minutes

   Because the relationship between the OD_{600} and the number of viable cells per milliliter varies substantially from strain to strain, it is essential to calibrate the spectrophotometer by measuring the OD_{600} of a growing culture of the particular strain of *E coli* at different times in its growth cycle and determining the number of viable cells at each of these times by plating dilutions of the culture on LB agar plates in the absence of antibiotics

4. Aseptically transfer the cells to sterile, disposable, ice-cold 50-ml polypropylene tubes (Falcon 2070). Cool the cultures to 0°C by storing the tubes on ice for 10 minutes

   Important: All subsequent steps in this procedure should be carried out aseptically

5. Recover the cells by centrifugation at 4000 rpm for 10 minutes at 4°C in a Sorvall GS3 rotor (or its equivalent).

6. Decant the media from the cell pellets Stand the tubes in an inverted position for 1 minute to allow the last traces of media to drain away.

7. Resuspend the pellets, by gentle vortexing, in approximately 20 ml (per 50-ml tube) of ice-cold transformation buffer (see Table 1 3 for TFB and Table 1 4 for FSB) (Use TFB when preparing competent cells that are to be used immediately and FSB when preparing competent cells that are to be stored at −70°C.) Store the resuspended cells on ice for 10 minutes.

8. Recover the cells by centrifugation at 4000 rpm for 10 minutes at 4°C in a Sorvall GS3 rotor (or its equivalent).

9. Decant the buffer from the cell pellets Stand the tubes in an inverted position for 1 minute to allow the last traces of buffer to drain away.

10. Resuspend the pellets by gentle vortexing in 4 ml (per 50-ml tube) of ice-cold TFB or FSB

TABLE 1.3 Preparation of 1 Liter of TFB

Reagent	Amount required/liter	Final concentration
1 M MES (pH 6.3)	10 ml	10 mM
$MnCl_2 \cdot 4H_2O$	8.91 g	45 mM
$CaCl_2 \cdot 2H_2O$	1.47 g	10 mM
KCl[a]	7.46 g	100 mM
Hexamminecobalt chloride	0.80 g	3 mM

1 M MES (2-[*N*-morpholino]ethanesulfonic acid, Ferguson et al. 1980) is prepared by dissolving 19.52 g of MES (Good and Izawa 1972) in 80 ml of pure H_2O (Milli-Q or equivalent). The pH of the solution is then adjusted to 6.3 with 5 M KOH, and pure H_2O is then added to bring the final volume to 100 ml. The solution is sterilized by filtration through a disposable Nalgene filter (0.45-micron pore size), divided into 10-ml aliquots, and stored frozen at −20°C.

Prepare the TFB by mixing all of the components with 800 ml of pure H_2O[b]. Adjust the volume of the final solution to 1 liter with pure H_2O. Sterilize the solution by filtration through a disposable Nalgene filter. Dispense the solution into 40-ml aliquots, and store the aliquots in tissue-culture flasks (Corning 25100 or equivalent) at 4°C. The pH of the solution should be between 6.0 and 6.1.

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[a] Rubidium chloride (100 mM) was included in the original recipe for TFB (Hanahan 1983). However, KCl has proved to be equally effective and is now recommended.

[b] Organic contaminants in the water used to prepare transformation buffers can reduce the efficiency of transformation of competent bacteria. Water obtained directly from a well-serviced Milli-Q filtration system (Millipore) usually gives good results. If problems should arise, the deionized water may be treated with activated charcoal.

TABLE 1.4 Preparation of 1 Liter of FSB

Reagent	Amount required/liter	Final concentration
1 M Potassium acetate (pH 7.5)	10 ml	10 mM
$MnCl_2 \cdot 4H_2O$	8.91 g	45 mM
$CaCl_2 \cdot 2H_2O$	1.47 g	10 mM
KCl	7.46 g	100 mM
Hexamminecobalt chloride	0.80 g	3 mM
Glycerol	100 ml	10%

1 M Potassium acetate is prepared by dissolving 9.82 g of potassium acetate in 90 ml of pure H_2O (Milli-Q or equivalent). The pH of the solution is then adjusted to 7.5 with 2 M acetic acid, and pure H_2O is then added to bring the final volume to 100 ml. The solution is divided into 10-ml aliquots, which are stored at −20°C.

Prepare the FSB by mixing all of the components with 800 ml of pure H_2O[a]. After the components are dissolved, adjust the pH of the solution to 6.4 with 0.1 N HCl. If you overshoot, do not readjust the pH by adding base; instead, discard the solution and begin again. Adjust the volume of the final solution to 1 liter with pure H_2O. Sterilize the solution by filtration through a disposable Nalgene filter (0.45-micron pore size). Dispense the solution into 40-ml aliquots and store the aliquots in tissue-culture flasks (Corning 25100 or equivalent) at 4°C. During storage, the pH of the solution will drift down to a final value of 6.1–6.2 and will then stabilize.

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[a] Organic contaminants in the water used to prepare transformation buffers can reduce the efficiency of transformation of competent bacteria. Water obtained directly from a well-serviced Milli-Q filtration system (Millipore) usually gives good results. If problems should arise, the deionized water may be treated with activated charcoal.

Proceed to prepare competent cells according to the procedure given in step 11a, for cells that are to be used immediately, or step 11b, for cells that are to be stored at −70°C and used at a later date.

11. a. To prepare fresh competent cells:

i Add 140 μl of DnD solution into the center of each suspension. Immediately mix the suspension by swirling gently, and then store it on ice for 15 minutes.

DnD solution contains

dithiothreitol	1 53 g
DMSO	9 ml
1 M potassium acetate (pH 7 5)	100 μl
H_2O to 10 ml	

Sterilize the DnD solution by filtration through a Millex SR membrane unit (Millipore), which is designed to withstand organic solvents Dispense 160-μl aliquots of the DnD solution into sterile 0.5-ml microfuge tubes. Close the tubes tightly and store them at −20°C.

Oxidation products of DMSO, presumably dimethyl sulfide, are inhibitors of transformation To avoid problems, purchase DMSO of the highest quality The contents of a fresh bottle should be divided into 10-ml aliquots in sterile tubes The tubes should be closed tightly and stored at −70°C Each aliquot should be used only once and discarded

For preparation of 1 M potassium acetate (pH 7 5), see Table 1 4

ii Add an additional 140 μl of DnD solution to each suspension Mix by swirling gently, and then store the suspension on ice for a further 15 minutes

iii Dispense aliquots of the suspensions into chilled, sterile polypropylene tubes (Falcon 2059, 17 mm × 100 mm) Store the tubes on ice For most cloning purposes, 50-μl aliquots of the competent-cell suspension will be more than adequate However, when large numbers of transformed colonies are required (e.g , when constructing cDNA libraries), larger aliquots may be needed.

After the addition of the DNA (step 12), the competent cultures are heated briefly to 42°C (step 13) This is a critical step, and it is important that the cells be raised to exactly the right temperature at the correct rate All of the times and temperatures given below have been worked out using Falcon 2059 tubes Other types of tubes will not necessarily yield equivalent results

b. To prepare frozen stocks of competent cells.

i. Add 140 μl of DMSO per 4 ml of resuspended cells Mix gently by swirling, and store the suspension on ice for 15 minutes

ii Add an additional 140 μl of DMSO to each suspension. Mix gently by swirling, and then return the suspensions to an ice bath.

iii. Working quickly, dispense aliquots of the suspensions into chilled, sterile microfuge tubes. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in liquid nitrogen Store the tubes at −70°C until needed For most cloning purposes, 50-μl aliquots of the competent-cell suspension will be more than adequate However, when large numbers of transformed colonies are required (e.g , when constructing cDNA libraries), larger aliquots may be needed

iv. When needed, remove a tube of competent cells from the −70°C freezer Thaw the cells by holding the tube in the palm of your hand. Just as the cells thaw, transfer the tube to an ice bath Store the cells on ice for 10 minutes

v Using a chilled, sterile pipette tip, transfer the competent cells to chilled, sterile polypropylene tubes (Falcon 2059, 17 mm × 100 mm) Store the cells on ice

After the addition of the DNA (step 12), the competent cultures are heated briefly to 42°C (step 13) This is a critical step, and it is important that the cells be raised to exactly the right temperature at the correct rate All of the times and temperatures given below have been worked out using Falcon 2059 tubes Other types of tubes will not necessarily yield equivalent results

12. Add the DNA to the competent cells, and swirl the tubes gently several times to mix their contents Store the tubes on ice for 30 minutes

For optimal results, the volume of the DNA solution should not exceed 5% of the volume of the competent cells The number of transformants increases in proportion to the amount of DNA added until the system becomes saturated Although there is some variation from one batch of competent cells to the next, 50-μl aliquots of competent cells are usually saturated by approximately 1 ng of supercoiled plasmid DNA Although more DNA can be added without affecting the total yield of transformants, the efficiency of the system (measured in transformants per microgram of DNA) is reduced when excess DNA is used This becomes important when the transforming DNA is in short supply (e g , cDNA synthesized from mRNA extracted from relatively inaccessible sources) To obtain the maximum number of transformed colonies, divide the available DNA among several aliquots of competent cells so that the system never becomes saturated

Be sure to include the following controls in your experiment
- Competent bacteria that receive a known amount of the standard preparation of supercoiled plasmid DNA
- Competent bacteria that receive no plasmid DNA at all

13. Transfer the tubes to a rack placed in a circulating water bath that has been preheated to 42°C Leave the tubes in the rack for exactly 90 seconds Do not shake the tubes

14. Rapidly transfer the tubes to an ice bath Allow the cells to chill for 1–2 minutes.

15. Add 800 μl of SOC medium (see Appendix A) to each tube. Warm the cultures to 37°C in a water bath, and then transfer the tubes to a shaking incubator set at 37°C. Incubate the cultures for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, the cells should be gently agitated (225 cycles/minute or less) during the recovery period.

16. Transfer the appropriate volume (up to 200 μl per 90-mm plate) of transformed competent cells onto agar SOB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic. Add additional broth if small volumes of culture (<10 μl) are transferred Using a sterile bent glass rod, gently spread the transformed cells over the surface of the agar plate

If more than 200 μl of transformed competent cells are to be plated on a single 90-mm plate, the cells should be concentrated by centrifugation (4000 rpm for 10 minutes at room temperature in a Sorvall SS34 rotor or its equivalent) and gently resuspended in an appropriate volume of SOC

The entire transformation mixture may be spread on a single plate (or plated in top agar) if resistance to tetracycline is used as the selectable marker However, if resistance to ampicillin is selected, only a portion of the culture (empirically determined) should be spread on a single plate The number of ampicillin-resistant colonies does not increase in linear proportion to the number of cells applied to the plate, perhaps because of growth-inhibiting substances released from the cells killed by the antibiotic

17. Leave the plates at room temperature until the liquid has been absorbed.

18. Invert the plates and incubate at 37°C Colonies should appear in 12–16 hours

When selecting for resistance to ampicillin, transformed cells should be plated at low density ($<10^4$ colonies per 90-mm plate) and the plates should not be incubated for more than 20 hours at 37°C β-Lactamase, secreted into the medium from ampicillin-resistant transformants, rapidly inactivates the antibiotic in regions surrounding the colonies Thus, plating cells at high density or incubating them for long periods results in the appearance of ampicillin-sensitive satellite colonies This problem is ameliorated, but not completely eliminated, by using carbenicillin rather than ampicillin in selective media and increasing the concentration of antibiotic from 60 μg/ml to 100 μg/ml

Note

Both Protocol I and Protocol II have been optimized for particular strains of *E. coli*; equivalent efficiencies of transformation may not be achieved with other strains

Protocol II: Fresh Competent E. coli Prepared Using Calcium Chloride

The following simple procedure is a variation of that of Cohen et al (1972) and is frequently used to prepare batches of competent bacteria that yield 5×10^6 to 2×10^7 transformed colonies per microgram of supercoiled plasmid DNA This efficiency of transformation is high enough to allow all routine cloning in plasmids to be performed with ease The procedure, which works well with most strains of *E coli*, is quick and is more reproducible than Protocol I. Competent cells made by this procedure may be preserved at −70°C, although there may be some deterioration in the efficiency of transformation during prolonged storage

1. Pick a single colony (2–3 mm in diameter) from a plate freshly grown for 16–20 hours at 37°C and transfer it into 100 ml of LB broth or SOB medium in a 1-liter flask. Incubate the culture for ~3 hours at 37°C with vigorous shaking (300 cycles/minute in a rotary shaker) For efficient transformation, it is essential that the number of viable cells should not exceed 10^8 cells/ml To monitor the growth of the culture, determine the OD_{600} every 20–30 minutes

   Because the relationship between the OD_{600} and the number of viable cells per milliliter varies substantially from strain to strain, it is essential to calibrate the spectrophotometer by measuring the OD_{600} of a growing culture of the particular strain of *E coli* at different times in its growth cycle and determining the number of viable cells at each of these times by plating dilutions of the culture on LB agar plates in the absence of antibiotics

2. Aseptically transfer the cells to sterile, disposable, ice-cold 50-ml polypropylene tubes (Falcon 2070) Cool the cultures to 0°C by storing the tubes on ice for 10 minutes

   Important: All subsequent steps in this procedure should be carried out aseptically.

3. Recover the cells by centrifugation at 4000 rpm for 10 minutes at 4°C in a Sorvall GS3 rotor (or its equivalent)

4. Decant the media from the cell pellets Stand the tubes in an inverted position for 1 minute to allow the last traces of media to drain away

5. Resuspend each pellet in 10 ml of ice-cold 0 1 M $CaCl_2$ and store on ice

   We find it convenient to store a 1 M stock solution of $CaCl_2$ (made in pure water [Milli-Q or equivalent]) in 10-ml aliquots at −20°C When preparing competent cells, thaw an aliquot and dilute it to 100 ml with pure water Sterilize the solution by filtration through a Nalgene filter (0 45-micron pore size), and then chill it to 0°C

   For most strains of *E coli* (except for MC1061), TFB (see Table 1 3) may be used at this stage instead of $CaCl_2$ with equivalent or better results

6. Recover the cells by centrifugation at 4000 rpm for 10 minutes at 4°C in a Sorvall GS3 rotor (or its equivalent).

7. Decant the fluid from the cell pellets, and stand the tubes in an inverted position for 1 minute to allow the last traces of fluid to drain away.

8. Resuspend each pellet in 2 ml of ice-cold 0.1 M CaCl_2 (or TFB, see Table 1.3 and notes to step 5) for each 50 ml of original culture.

   At this point, the cells can be dispensed into aliquots that can be frozen at −70°C (for instructions, see step 11b, iii, on page 1.80). The cells maintain competency under these conditions, although the transformation efficiency may drop slightly during prolonged storage.

   Dagert and Ehrlich (1979) have shown that cells may be stored at 4°C in CaCl_2 solution for 24–48 hours. The efficiency of transformation increases four- to sixfold during the first 12–24 hours of storage and then decreases to the original level.

9. Using a chilled, sterile pipette tip, transfer 200 μl of each suspension of competent cells to a sterile microfuge tube. Add DNA (no more than 50 ng in a volume of 10 μl or less) to each tube. Mix the contents of the tubes by swirling gently. Store the tubes on ice for 30 minutes.

   Be sure to include the following controls in your experiment:
   - Competent bacteria that receive a known amount of the standard preparation of supercoiled plasmid DNA
   - Competent bacteria that receive no plasmid DNA at all

10. Transfer the tubes to a rack placed in a circulating water bath that has been preheated to 42°C. Leave the tubes in the rack for exactly 90 seconds. Do not shake the tubes.

11. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1–2 minutes.

12. Add 800 μl of SOC medium (see Appendix A) to each tube. Incubate the cultures for 45 minutes in a water bath set at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

    If higher transformation efficiency is required, agitate the cells gently (225 cycles/minute or less in a rotary shaker) at 37°C during the recovery period.

13. Transfer the appropriate volume (up to 200 μl per 90-mm plate) of transformed competent cells onto agar SOB medium containing 20 mM MgSO_4 and the appropriate antibiotic. Add additional broth if small volumes of culture (<10 μl) are transferred. Using a sterile bent glass rod, gently spread the transformed cells over the surface of the agar plate.

    If more than 200 μl of transformed competent cells are to be plated on a single 90-mm plate, the cells should be concentrated by centrifugation and gently resuspended in an appropriate volume of SOC.

    The entire transformation mixture may be spread on a single plate (or plated in top agar) if resistance to tetracycline is used as the selectable marker. However, if resistance to ampicillin is selected, only a portion of the culture (empirically determined) should be spread on a single plate. The number of ampicillin-resistant colonies does not increase in linear proportion to the number of cells applied to the plate, perhaps because of growth-inhibiting substances released from the cells killed by the antibiotic.

14. Leave the plates at room temperature until the liquid has been absorbed

15. Invert the plates and incubate at 37°C Colonies should appear in 12–16 hours.

When selecting for resistance to ampicillin, transformed cells should be plated at low density (<10^4 colonies per 90-mm plate) and the plates should not be incubated for more than 20 hours at 37°C β-Lactamase, secreted into the medium from ampicillin-resistant transformants, rapidly inactivates the antibiotic in regions surrounding the colonies Thus, plating cells at high density or incubating them for long periods results in the appearance of ampicillin-sensitive satellite colonies This problem is ameliorated, but not completely eliminated, by using carbenicillin rather than ampicillin in selective media and increasing the concentration of antibiotic from 60 μg/ml to 100 μg/ml

Note

Both Protocol I and Protocol II have been optimized for particular strains of *E coli*, equivalent efficiencies of transformation may not be achieved with other strains

Identification of Bacterial Colonies That Contain Recombinant Plasmids

There are four methods that are commonly used to identify bacterial colonies that contain recombinant plasmids:

- Restriction analysis of small-scale preparations of plasmid DNA
- α-Complementation
- Insertional inactivation
- Screening by hybridization

RESTRICTION ANALYSIS OF SMALL-SCALE PREPARATIONS OF PLASMID DNA

In this method, a number of independently transformed bacterial colonies are picked and grown in small-scale cultures. Plasmid DNAs, isolated from each culture by one of the methods described earlier in this chapter, are then analyzed by digestion with restriction enzymes and gel electrophoresis. This procedure, although laborious, is the method of choice when there is a high chance of finding the desired recombinant within a small number of randomly chosen transformed colonies, for example, when ligation of the vector to the segment of foreign DNA yields a significantly higher number of transformed colonies than either of the control ligations (vector alone and foreign fragment alone). With practice, it is possible to extract and analyze 36 minipreparations of plasmid DNA in 2–3 hours.

α-COMPLEMENTATION

Many of the vectors in current use (e.g., the pUC series) carry a short segment of *E. coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lacZ*). Embedded in this coding region is a polycloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of β-galactosidase. Vectors of this type are used in host cells that code for the carboxy-terminal portion of β-galactosidase. Although neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to form an enzymatically active protein. This type of complementation, in which deletion mutants of the operator-proximal segment of the *lacZ* gene are complemented by β-galactosidase-negative mutants that have the operator-proximal region intact, is called α-complementation (Ullmann et al. 1967). The Lac$^+$ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Horwitz et al. 1964). However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in production of an amino-terminal fragment that is not capable of α-complementation Bacteria carrying recombinant plasmids therefore form white colonies. The development of this simple color test has greatly simplified the identification of recombinants constructed in plasmid vectors of this type It is easily possible to screen many thousands of colonies visually and to recognize colonies that carry putative recombinant plasmids The structure of these plasmids is then verified by restriction analysis of minipreparations of plasmid DNA

Testing Bacteria for α-Complementation

1. To a premade LB agar plate containing the appropriate antibiotics, add 40 μl of a stock solution of X-gal (20 mg/ml in dimethylformamide) and 4 μl of a solution of isopropylthio-β-D-galactoside (IPTG) (200 mg/ml)

   X-gal is very expensive However, the cost can be minimized by spreading a concentrated solution of X-gal on the surface of a premade agar plate rather than incorporating the sugar throughout the entire volume of the agar medium

   A stock solution of X-gal is prepared by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution Use a glass or polypropylene tube The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and should be stored at −20°C It is not necessary to sterilize X-gal solutions by filtration

   IPTG solution is prepared by dissolving 2 g of IPTG in 8 ml of H_2O Adjust the volume of the solution to 10 ml with H_2O and sterilize by filtration through a 0 22-micron disposable filter Dispense the solution into 1-ml aliquots and store them at 20°C

2. Using a sterile glass spreader (or a bent pasteur pipette whose tip has been sealed in a flame), spread the solution over the entire surface of the plate. Incubate the plate at 37°C until all of the fluid has disappeared Because of the low volatility of dimethylformamide, this can take up to 3 or 4 hours if the plate is freshly made

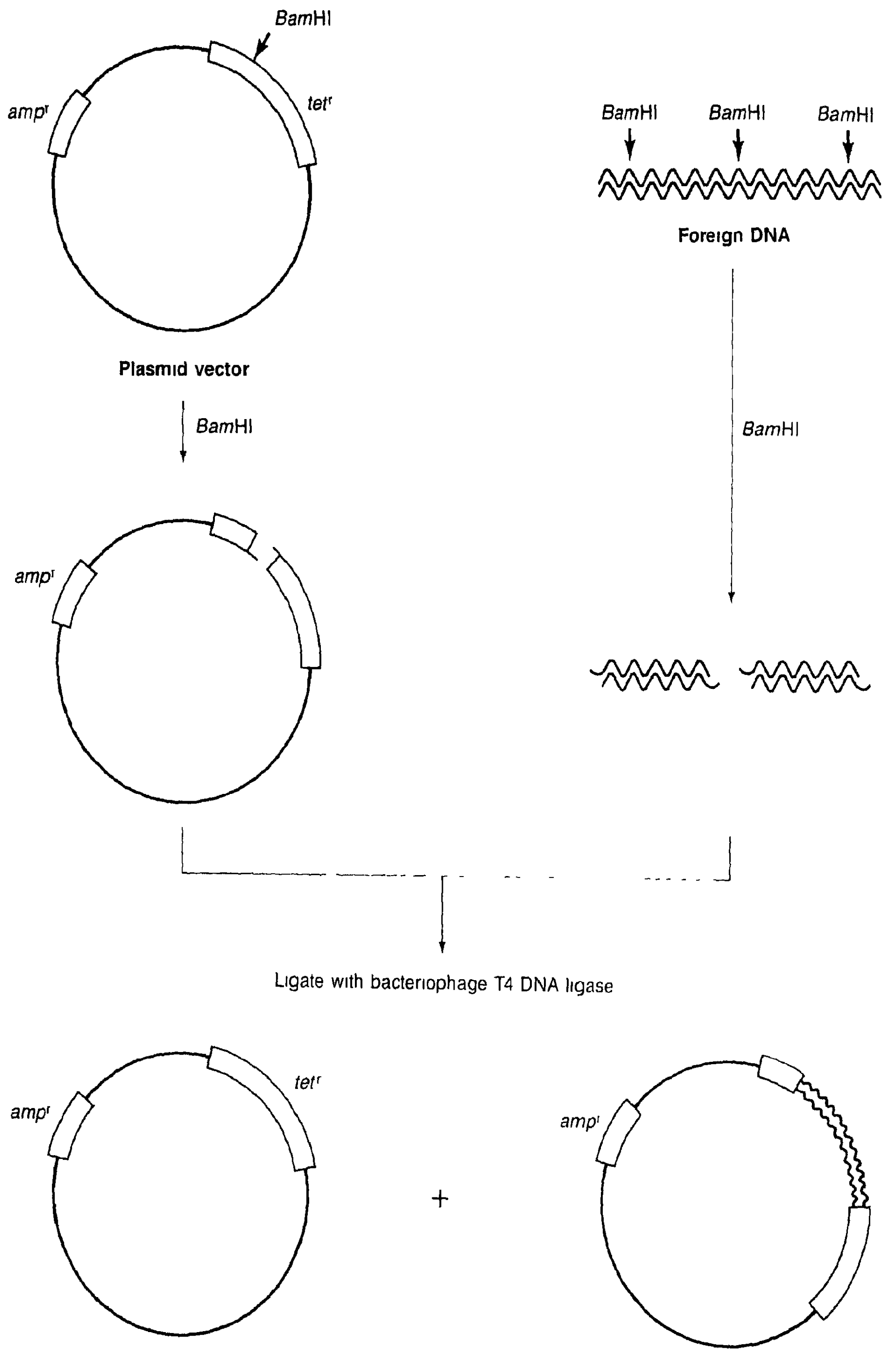
3. Inoculate the plate with the bacteria that are to be tested This can be done by streaking with a bacterial loop or toothpicks or by spreading up to 100 μl of a suspension of bacteria over the surface of the agar medium After the inoculum has absorbed, incubate the plate in an inverted position for 12–16 hours at 37°C

4. Store the plate at 4°C for several hours This allows the blue color to develop fully Colonies that contain active β-galactosidase are pale blue in the center and dense blue at their periphery White colonies occasionally show a faint blue spot in the center, but these are colorless at the periphery.

INSERTIONAL INACTIVATION

This method, which is shown in Figure 1.12, can only be used with older vectors (e.g., pBR322) that carry two or more antibiotic resistance genes and an appropriate distribution of restriction enzyme cleavage sites. The DNA to be inserted and the purified plasmid DNA are digested with restriction enzymes that recognize sites located in only one of the antibiotic resistance genes (in this example, the *tet*^{r} gene). After ligating the two DNAs at the appropriate concentrations, the ligation mixture is used to transform *E. coli*. Transformants are selected that are resistant to the second antibiotic (in this example, ampicillin). Some of the colonies that grow in the presence of ampicillin will contain recombinant plasmids, others will contain plasmid DNA that has recircularized during ligation without insertion of the foreign DNA. To discriminate between the two kinds of transformants, separate plates containing ampicillin and tetracycline are inoculated with a number of colonies in patches in identical locations (Figure 1.13). The colonies that survive and grow in the presence of tetracycline contain plasmids with active *tet*^{r} genes, and it is unlikely that such plasmids carry insertions of foreign DNA. The colonies that do not grow in the presence of tetracycline but do grow on the plate containing ampicillin contain plasmids with inactive *tet*^{r} genes, and it is likely that these plasmids carry the foreign DNA sequences.

Although insertion of foreign DNA sequences within an antibiotic resistance gene almost always leads to inactivation of that gene, at least one case has been reported in which an insertion left the gene in a functional state. Villa-Komaroff et al. (1978) found that insertion of a segment of rat preproinsulin cDNA into the *Pst*I site of pBR322 did not inactivate the *amp*^{r} gene. Presumably, a small piece of foreign DNA had been inserted that did not alter the reading frame of the *amp*^{r} gene, so that a fusion protein was formed that retained β-lactamase activity.



Reconstitution of the original *Bam*HI site without insertion of a segment of foreign DNA restores the *tet*ʳ gene Bacteria transformed with this non-recombinant plasmid can grow in the presence of ampicillin or tetracycline

Insertion of a segment of foreign DNA inactivates the *tet*ʳ gene Bacteria transformed with this recombinant plasmid can grow in the presence of ampicillin but not tetracycline

FIGURE 1.12
Insertional inactivation.

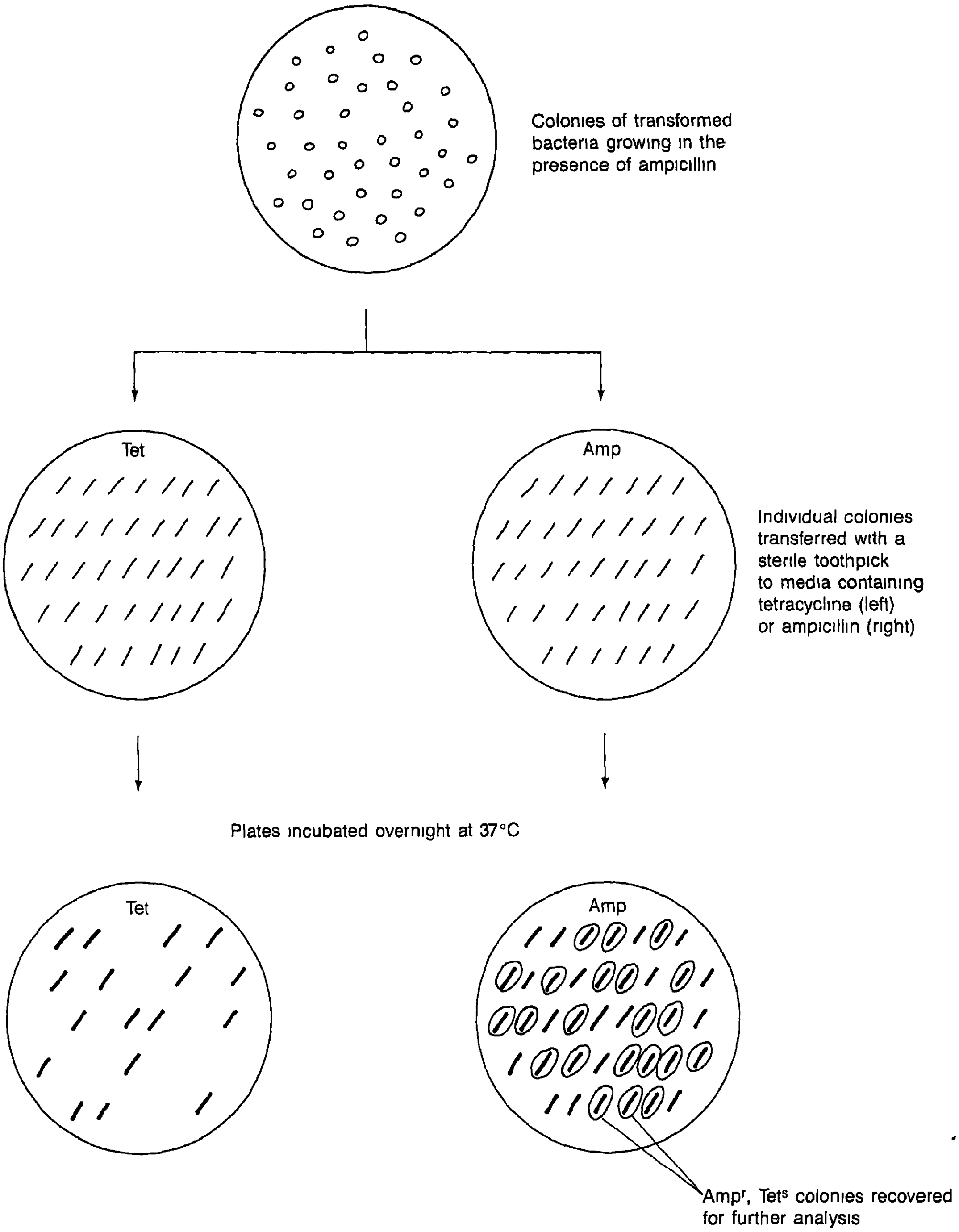

Colonies of transformed bacteria growing in the presence of ampicillin
Tet
Amp
Individual colonies transferred with a sterile toothpick to media containing tetracycline (left) or ampicillin (right)
Plates incubated overnight at 37°C
Tet
Amp
Amp^r, Tet^s colonies recovered for further analysis

FIGURE 1.13
Screening for insertions of foreign DNA by inactivation of plasmid-borne antibiotic resistance genes

SCREENING BY HYBRIDIZATION

In 1975, Grunstein and Hogness described a method for in situ lysis of bacterial colonies on nitrocellulose filters and noncovalent attachment of the released DNA to the filters. The DNA could then be hybridized to appropriate radiolabeled nucleic acid probes. This procedure was an extension of the work of Nygaard and Hall (1963), who had shown that DNA could be immobilized on nitrocellulose, and Denhardt (1966) and Gillespie and Spiegelman (1965), who were the first to demonstrate that such fixed nucleic acids could be detected by hybridization. Although minor modifications have been introduced over the years, the protocol originally described by Grunstein and Hogness has proved to be remarkably durable. It remains the most commonly used technique to identify individual bacterial colonies carrying cosmids or plasmids that contain DNA sequences of interest. Methods are presented below to screen bacterial colonies with radiolabeled probes that are longer than 100 nucleotides in length. The preparation of these ^{32}P-labeled probes is described in Chapter 10. Methods for screening bacterial colonies with shorter radiolabeled oligonucleotides are given in Chapter 11. It is easily possible to screen many hundreds of thousands of colonies simultaneously and to recognize colonies that carry recombinant plasmids containing the target sequences. The structure of these plasmids is then verified by restriction analysis and Southern hybridization of minipreparations of plasmid DNA.

Three types of solid supports may be used for in situ hybridization of lysed bacterial colonies—nitrocellulose filters, nylon membranes, and Whatman 541 filter papers. *Nylon membranes* are the most durable of the three and will withstand several rounds of hybridization and washing at elevated temperatures. They are therefore preferred when colonies are to be screened sequentially with a number of different probes. Because different brands of nylon membranes require different treatments, we recommend following the manufacturer's instructions precisely during immobilization and hybridization of DNA. *Whatman 541* filter paper, which has a high wet strength, was first used by Gergen et al. (1979) to screen bacterial colonies. Whatman 541 paper has been used chiefly to screen libraries that have been arranged and stored as cultures of individual colonies in separate wells of microtiter plates (Linbro Scientific). These ordered libraries are duplicated on the surface of agar medium (usually in square petri dishes), and the resulting colonies are then transferred to Whatman 541 paper and lysed either by alkali or by a combination of alkali and heat (Maas 1983). Conditions for hybridization of the immobilized DNA are essentially identical to those established for nitrocellulose filters. Whatman 541 paper has some advantages over nitrocellulose filters. It is cheaper, more durable during hybridization, and less prone to distortion and cracking during drying. However, unless care is taken during the denaturation step (Maas 1983), the strength of the hybridization signal is significantly lower than that obtained from nitrocellulose filters. For routine screening of bacterial colonies, therefore, *nitrocellulose filters* remain the preferred choice as solid supports.

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on filters. These methods differ in the following respects:

- Solvent and temperature used (e g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for times as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or poly-ethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

These and other factors affecting hybridization of immobilized nucleic acids are discussed in detail in Chapter 9, pages 9 47–9 51.

Transferring Small Numbers of Colonies to Nitrocellulose Filters

This procedure is used when it is necessary to screen a small number of bacterial colonies (100–200) that are dispersed over several agar plates. The colonies are consolidated on a master agar plate and on a nitrocellulose filter laid on the surface of a second agar plate. After a period of growth, the colonies are lysed in situ. The master plate is stored at 4°C until the results of the screening procedure become available.

1. Place a nitrocellulose filter (Millipore HAWP or equivalent) on an agar plate containing the selective antibiotic.

   It is not necessary to use a detergent-free or sterilized filter in this procedure; such filters are required only when small numbers of bacteria are used as inocula, for example, when the transformation mixture is plated directly on the surface of the filter. However, the filter should be handled with gloved hands, since finger oils prevent wetting of the filter and affect DNA transfer.

2. Using sterile toothpicks, transfer individual bacterial colonies onto the filter and then onto a master agar plate that contains the selective antibiotic but no filter. Make small streaks 2–3 mm in length (or dots) arranged in a grid pattern. Each colony should be streaked in an identical position on both plates. Up to 100 colonies can be streaked onto a single 90-mm plate. Finally, streak a colony containing a nonrecombinant plasmid (e.g., pBR322) onto both the filter and the master plate. This negative control is often useful and sometimes necessary to discriminate between specific annealing of the radioactive probe to a recombinant plasmid and nonspecific background hybridization.

3. Invert the plates and incubate them at 37°C until the bacterial streaks have grown to a width of 0.5–1.0 mm.

   At this stage, when the bacteria are still growing rapidly, the filter may be transferred to an agar plate containing chloramphenicol (170–200 μg/ml) and incubated for 12 hours at 37°C (Hanahan and Meselson 1980, 1983). This amplification step is necessary only when the copy number of the recombinant plasmid is expected to be low (e.g., if an unusually large segment [>10 kb] of foreign DNA has been inserted) or when highly degenerate oligonucleotides are to be used as probes. Under normal circumstances, cloned DNA sequences can be detected very easily by hybridization without prior amplification of the recombinant plasmid. Amplification can be carried out only with vectors that replicate in a relaxed fashion (see page 1.3).

4. Mark the nitrocellulose filter in three or more asymmetric locations by stabbing through it and into the agar beneath with an 18-gauge needle attached to a syringe containing waterproof black drawing ink. Mark the master plate in approximately the same locations.

   With practice, it is possible to avoid the use of ink (which can be messy) by punching holes through the filter into the underlying agar with an empty 18-gauge needle. After hybridization, backlighting can be used to align the holes in the filter with the marks in the agar.

5. Seal the master plate with Parafilm and store it at 4°C in an inverted position until the results of the hybridization reaction are available.

6. Lyse the bacteria and bind the liberated DNA to the nitrocellulose filter by one of the two procedures described on pages 1.98 and 1.100.

Replicating Colonies onto Nitrocellulose Filters

Two methods are given here for replicating colonies onto nitrocellulose filters. Method 1 (Hanahan and Meselson 1980, 1983) is used when it is necessary to screen large numbers of colonies by hybridization (e.g., when plating out bacteria transformed by a cDNA library constructed in a plasmid vector). In this case, bacteria are plated directly from a transformation mixture onto detergent-free nitrocellulose filters, and replica filters are prepared by filter-to-filter contact. Method 2 is used to transfer many bacterial colonies simultaneously from the surfaces of agar plates to nitrocellulose filters. This method works with bacterial colonies of any size, but small colonies (0.1–0.2 mm) give the best results; they produce sharper hybridization signals and smear less than larger colonies. As many as 2×10^4 colonies per 138-mm filter or 10^4 colonies per 82-mm filter can be screened using this technique.

METHOD 1

1. Number dry, detergent-free nitrocellulose filters (Millipore HATF or equivalent) with a soft-lead pencil or a ballpoint pen, wet them with water, and sandwich them between dry Whatman 3MM papers. Wrap the stack of filters in aluminum foil, and sterilize them by autoclaving (15 lb/sq. in. on liquid cycle). Prepare enough filters to make a master and two replicas of each plate.

2. Using sterile, blunt-ended forceps (e.g., Millipore forceps), lay a sterile filter, numbered side down, on a day-old LB (or SOB) agar plate containing the appropriate antibiotic. When the filter is thoroughly wet, peel it from the plate and replace it, numbered side up, on the surface of the agar.

3. Apply the bacteria, in a small volume of liquid, to the center of the filter on the surface of the agar plate. A large filter (138-mm diameter) will accommodate up to 0.5 ml of liquid containing 2×10^4 viable bacteria. A small filter (82-mm diameter) will accommodate up to 0.2 ml of liquid containing approximately 10^4 bacteria. Using a sterile glass spreader, disperse the fluid evenly over the surface of the filter. Leave a border 2–3 mm wide at the edge of the filter free of bacteria. After spreading the inoculum, incubate the plate (noninverted) with the lid ajar for a few minutes in a laminar flow hood to allow the inoculum to evaporate. Then close the lid, invert the plate, and incubate at 37°C until small colonies (0.1–0.2-mm diameter) appear (about 8–10 hours).

4. If desired, replica filters may be prepared at this stage (see step 6). Otherwise, the filter (colony side up) should be transferred to an LB (or SOB) agar plate containing the appropriate antibiotic and 25% glycerol (Hanahan and Meselson 1983). Incubate the plate for 2 hours at 37°C.

5. Seal the plate well with Parafilm, and store it in an inverted position in a sealed plastic bag at −20°C. Replicas can be made after thawing the master plate at room temperature (still in the inverted position).

6. Replica filters are made as follows:

a. Prepare in advance a stack of Whatman 3MM papers (one for each filter plus a few spares) cut to a size slightly larger than the filters Sterilize the stack of 3MM papers by autoclaving for 10 minutes at 10 lb/sq. in. on liquid cycle

b. Peel the master nitrocellulose filter off the storage plate and lay it, colony side up, on a dampened pad of sterile 3MM paper

c. Number a damp, sterile nitrocellulose filter and lay it on the master nitrocellulose filter. Take care to prevent air bubbles from becoming trapped between the two filters This is best done by bending the second filter slightly so that contact is first made between the centers of the filters Be careful not to move the filters relative to one another once contact has been made Try to arrange the filters so that they do not overlap exactly; this makes it easier to separate the two filters

d. Press the two filters firmly together with a velvet replica-plating tool (Figure 1.14) or with a heavy glass plate (with a 3MM paper between the glass and the filters)

e Orient the two filters by making a series of holes in the pair of filters with an 18-gauge needle

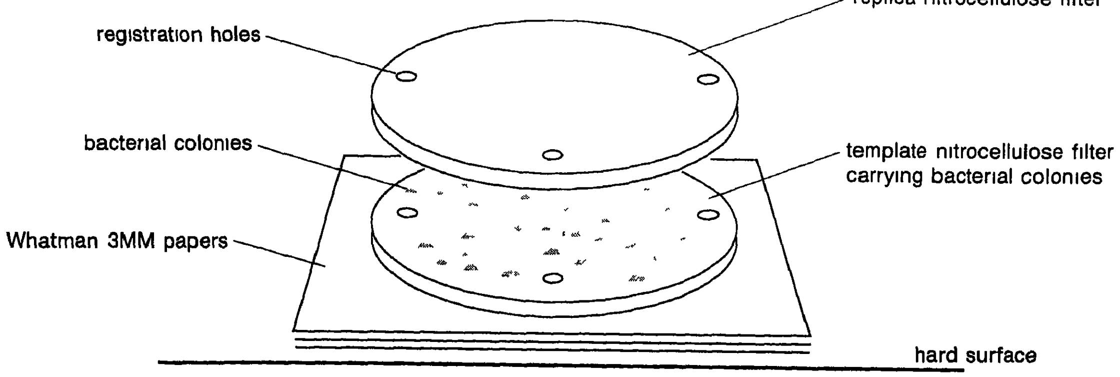


FIGURE 1.14
Use of a replica-plating tool to replicate bacterial colonies growing on nitrocellulose filters.

f Peel the filters apart. Lay the replica on a fresh LB (or SOB) agar plate containing the appropriate antibiotic and incubate the plate at 37°C until colonies appear (4–6 hours).

At this stage, when the bacteria are still growing rapidly, the filter may be transferred to an agar plate containing chloramphenicol (170–200 μg/ml) and incubated for 12 hours at 37°C (Hanahan and Meselson 1980, 1983) This amplification step is necessary only when the copy number of the recombinant plasmid is expected to be low (e g , if an unusually large segment [> 10 kb] of foreign DNA has been inserted) or when highly degenerate oligonucleotides are to be used as probes Under normal circumstances, cloned DNA sequences can be detected very easily by hybridization without prior amplification of the recombinant plasmid Amplification can be carried out only with vectors that replicate in a relaxed fashion (see page 1 3)

g Replace the master nitrocellulose filter on a fresh LB (or SOB) agar plate containing the appropriate antibiotic and 25% glycerol (Hanahan and Meselson 1983). Incubate the plate for 1 hour at 37°C, and then freeze it as described in step 5.

If necessary, more replicas can be prepared from the master in an identical manner Key the second and subsequent replicas to the existing holes in the master filter Return both filters to their respective plates If the master filter is to be used to make more than two replicas, it should be incubated for a few hours to allow the colonies to regenerate Generally, making two to three replicas presents no problem However, should more replicas be required, it is essential that the bacterial colonies be extremely small (~0 1-mm diameter) at the time of replication in order to avoid smearing To avoid contamination, filters containing copies of valuable libraries are best handled in a laminar flow hood

7. Lyse the bacteria and bind the liberated DNA to the replica filters using one of the two methods described on pages 1.98 and 1.100.

METHOD 2

1. Plate the bacterial suspension directly on the surfaces of LB (or SOB) agar plates containing the appropriate antibiotic Plates that are 2–3 days old give the best results because they absorb the inoculum more readily Leave the plates, with their lids ajar, in a laminar flow hood until the surface of the agar is quite dry. Close the lids and incubate the plates in an inverted position for 12–14 hours at 37°C Chill the plates for 30–60 minutes at 4°C.

2. Number a dry, detergent-free nitrocellulose filter (Millipore HATF or equivalent) with a soft-lead pencil or a ballpoint pen, and sterilize it as described in step 1, page 1 93 Place the filter, numbered side down, on the surface of the LB (or SOB) agar medium, in contact with the bacterial colonies, until it is completely wet Mark the filter and the underlying medium in three or more asymmetric locations by stabbing through the filter with an 18-gauge needle attached to a syringe containing waterproof black drawing ink

   Although sterile filters are preferred, nonsterile filters can also be used as long as no more replicas are to be made from the master plate

   With practice, it is possible to avoid the use of ink (which can be messy) by punching holes through the filter into the underlying agar with an empty 18-gauge needle After hybridization, backlighting can be used to align the holes in the filter with the marks in the agar

3. Using blunt-ended forceps (e g , Millipore forceps), peel off the filter

4. At this stage, several options are available

   - The bacteria adhering to the filter may be immediately lysed and the liberated DNA bound to the filter using one of the two methods described on pages 1 98 and 1 100
   - The filter may be placed, colony side up, on the surface of a fresh LB (or SOB) agar plate containing the appropriate antibiotic The colonies are lysed after a few hours of incubation, when they have grown to a size of 2–3 mm This method is necessary only when the transfer of the colonies to the filter is poor or uneven, which is not usually the case
   - The filter may be transferred to an agar plate containing chloramphenicol (170–200 μg/ml) and incubated for 12 hours at 37°C (Hanahan and Meselson 1980, 1983) This amplification step is necessary only when the copy number of the recombinant plasmid is expected to be low (e g., if an unusually large segment [> 10 kb] of foreign DNA has been inserted) or when highly degenerate oligonucleotides are to be used as probes. Under normal circumstances, cloned DNA sequences can be detected very easily by hybridization without prior amplification of the recombinant plasmid Amplification can be carried out only with vectors that replicate in a relaxed fashion (see page 1.3).
   - The filter may be used to prepare a second replica. The filter is placed, colony side up, on the surface of a fresh LB (or SOB) agar plate

containing the appropriate antibiotic. A second dry nitrocellulose filter is then laid carefully on top of the first and keyed to it. The filter sandwich is incubated for several hours at 37°C, and the plasmids are amplified, if desired, by further incubation on an agar plate containing chloramphenicol. The filters are kept as a sandwich during the subsequent lysis and neutralization steps but are peeled apart before the final wash (Ish-Horowicz and Burke 1981).

5. Incubate the master plate for 5–7 hours at 37°C until the colonies have regenerated Seal the plate with Parafilm, and store it at 4°C in an inverted position.

Lysis of Colonies and Binding of DNA to Nitrocellulose Filters

Two methods (based on the original procedure of Grunstein and Hogness [1975]) are described to liberate the DNA from bacterial colonies and to bind it to the nitrocellulose filter in situ. The first method can easily be adapted to accommodate large batches of filters, whereas the second is more economical when dealing with only one or two filters In both cases, try to avoid getting any of the solutions onto the upper surface of the filter and trapping air bubbles under the filter

METHOD 1

1. Cut four pieces of Whatman 3MM paper to an appropriate size and shape and fit them neatly onto the bottoms of four glass or plastic trays Cafeteria trays are ideal for this purpose, since they can accommodate batches of up to 25 filters Saturate each of the pieces of 3MM paper with one of the following solutions

   - 10% SDS (optional)
   - Denaturing solution (0 5 N NaOH, 1 5 M NaCl)
   - Neutralizing solution (1.5 M NaCl, 0 5 M Tris Cl [pH 7 4])
   - 2× SSC

   Pour off any excess liquid

   If the 3MM paper is too wet, the bacterial colonies swell and diffuse during lysis The hybridization signals then become blurred and attenuated, and it is very difficult to identify individual colonies that give rise to the signal

2. Using blunt-ended forceps (e g , Millipore forceps), peel the nitrocellulose filters from their plates and place them, colony side up, on the SDS-impregnated 3MM paper This treatment limits the diffusion of the plasmid DNA during denaturation and neutralization, resulting in a sharper hybridization signal

3. After the first filter has been exposed to the SDS solution for 3 minutes, transfer it to the second sheet of 3MM paper, which has been saturated with denaturing solution Transfer the remainder of the filters in the same order that they were removed from their agar plates Expose each filter to the denaturing solution for 5 minutes

   When transferring filters from one tray to another, use the edge of the first tray as a scraper to remove as much fluid as possible from the underside of the filter Alternatively, remove excess liquid by transferring the filter briefly to a dry paper towel

   Try to avoid getting fluid on the side of the filter carrying the bacterial colonies

4. Transfer the filters to the third sheet of 3MM paper, which has been saturated with neutralizing solution. Leave the filters for 5 minutes.

5. Transfer the filters to the last sheet of 3MM paper, which has been saturated with 2× SSC Leave the filters for 5 minutes.

6. Lay the filters, colony side up, on a sheet of dry 3MM paper. Allow them to dry at room temperature for at least 30 minutes

7. Sandwich the filters between two sheets of dry 3MM paper. Fix the DNA to the filters by baking for 1–2 hours at 80°C in a vacuum oven.

   Overbaking can cause the filters to become brittle Filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically

8. Hybridize the DNA immobilized on the filters to a ^{32}P-labeled probe as described on pages 1 101–1.104.

   Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature

METHOD 2

1. For each filter, make a puddle (0.75 ml) of 0.5 N NaOH on a piece of Saran Wrap. Place a filter, colony side up, on the puddle, stretching the Saran Wrap so that the filter wets evenly. Leave the filter in place for 2–3 minutes.

2. Blot the underside of the filter on a dry paper towel and repeat step 1 using a fresh piece of Saran Wrap and fresh 0.5 N NaOH.

3. Blot the filter again and transfer it to a puddle of 1 M Tris·Cl (pH 7.4) on a fresh piece of Saran Wrap. After 5 minutes, blot the filter and repeat the step using a fresh piece of Saran Wrap and fresh 1 M Tris·Cl (pH 7.4).

4. Blot the filter and transfer it to a puddle of 1.5 M NaCl, 0.5 M Tris·Cl (pH 7.4) on a fresh piece of Saran Wrap. After 5 minutes, blot the filter and transfer it to a piece of dry 3MM paper. Allow the filter to dry at room temperature for 20–30 minutes.

5. Sandwich the filter between two sheets of dry 3MM paper. Fix the DNA to the filter by baking for 2 hours at 80°C in a vacuum oven.

   Overbaking can cause the filters to become brittle. Filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

6. Hybridize the DNA immobilized on the filter to a ^{32}P-labeled probe as described on pages 1.101–1.104.

   Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

Hybridization to Nitrocellulose Filters Containing Replicas of Bacterial Colonies

The following protocol is designed for 30 circular nitrocellulose filters, 82 mm in diameter. Appropriate adjustments to the volumes should be made when carrying out hybridization reactions with different numbers or sizes of filters.

1. Float the baked filters on the surface of a tray of 2× SSC until they have become thoroughly wetted from beneath Submerge the filters for 5 minutes

2. Transfer the filters to a glass crystallizing dish containing at least 200 ml of prewashing solution Stack the filters on top of one another in the solution Cover the dish with Saran Wrap and transfer it to a rotating platform in an incubator. In this and all subsequent steps, the filters should be slowly agitated to prevent them from sticking to one another. Incubate the filters for 30 minutes at 50°C.

   Important: Do not allow the filters to dry at any stage during the prewashing, prehybridization, or hybridization steps

   Prewashing solution

   5× SSC
   0.5% SDS
   1 mM EDTA (pH 8.0)

3. Gently scrape the bacterial debris from the surfaces of the filters using Kimwipes soaked in prewashing solution This reduces background hybridization without affecting the intensity or sharpness of positive signals

4. Transfer the filters to 150 ml of prehybridization solution in a glass crystallizing dish. Incubate the filters for 1–2 hours at the appropriate temperature (i e , 68°C when hybridization is to be carried out in aqueous solution, 42°C when hybridization is to be carried out in 50% formamide)

   Some workers prefer to incubate filters in heat-sealable plastic bags (Sears Seal-A-Meal or equivalent) (see, e g , Chapter 9, page 9 53) This method avoids problems with evaporation and, because the sealed bags can be submerged in a water bath, ensures that the temperatures during hybridization and washing are correct The bags must be opened and resealed when changing buffers To avoid radioactive contamination of the water bath, the resealed bag containing radioactivity should be sealed inside a second, noncontaminated bag.

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases It is important that these bubbles be removed by occasionally agitating the fluid in the bag, otherwise, the components of the prehybridization solution will not be able to coat the filter evenly This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag

   The filters should be completely covered by the prehybridization solution During prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become blocked by proteins in the BLOTTO

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A) at a concentration of 1 μg/ml should be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference Both versions of these solutions give excellent results and neither has clear-cut advantages over the other However, hybridization in 50% formamide at 42°C is less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution Offsetting this advantage is the two- to threefold slower rate of hybridization in solutions containing formamide

To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below T_m (see Chapter 9, pages 9 50–9 51) Both solutions work equally well when hybridization is carried out in aqueous solvents However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power

Prehybridization solution

Either
50% formamide
6× SSC (or 6× SSPE)
0.05× BLOTTO

or
6× SSC (or 6× SSPE)
0.05× BLOTTO

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No 1 paper Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

1 × BLOTTO: Bovine Lacto Transfer Technique Optimizer (Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide It should be stored at 4°C 1× BLOTTO is as effective a blocking agent as Denhardt's reagent, but much less expensive. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent when radiolabeled RNA is used as the hybridization probe, because of the possibility that the dried milk may contain significant amounts of RNAase activity.

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

5. Denature ^{32}P-labeled double-stranded DNA probe by heating for 5 minutes to 100°C. Chill the probe rapidly in ice water. Single-stranded probe need not be denatured. Add the probe to the prehybridization solution covering the filters. Incubate at the appropriate temperature until $1\text{–}3 \times C_0t_{1/2}$ is achieved (see Chapter 9, page 9.48). During the hybridization, the containers holding the filters should be tightly closed to prevent the loss of fluid by evaporation.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   Between 2×10^5 and 1×10^6 cpm of ^{32}P-labeled probe (sp. act. $\geq 5 \times 10^7$ cpm/μg) should be used per milliliter of prehybridization solution. Using more probe will cause the background of nonspecific hybridization to increase, using less will reduce the rate of hybridization.

6. When the hybridization is completed, remove the hybridization solution and immediately immerse the filters in a large volume (300–500 ml) of 2× SSC and 0.1% SDS at room temperature. Agitate the filters gently, and turn them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more. At no stage during the washing procedure should the filters be allowed to dry.

   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment. However, hybridization mixtures containing complementary strands of DNA should be discarded since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization.

7. Wash the filters twice for 1–1.5 hours in 300–500 ml of a solution of 1× SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of a solution of 0.2× SSC and 0.1% SDS at 68°C.

8. Dry the filters in the air at room temperature on paper towels. Arrange the filters (numbered side up) on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filters. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

9. Cover the filters with a second sheet of Saran Wrap Expose the filters to X-ray film (Kodak XAR-2 or equivalent) for 12–16 hours at −70°C with an intensifying screen (see Appendix E)

10. Develop the film and align it with the filters using the marks left by the radioactive ink. Use a nonradioactive fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters Tape a piece of clear Mylar or other firm transparent sheet to the film Mark on the clear sheet the positions of positive hybridization signals Also mark (in a different color) the positions of the asymmetrically located dots Remove the clear sheet from the film Identify the positive colonies by aligning the dots on the clear sheet with those on the agar plate.

    Some batches of nitrocellulose filters swell and distort during hybridization and subsequent drying, so that it becomes difficult to align the two sets of dots This problem can be alleviated to some extent by autoclaving the dry filters between pieces of damp Whatman 3MM paper before use (10 lb/sq in for 10 minutes on liquid cycle) Nylon membranes do not suffer from this problem

11. Using a sterile toothpick, transfer each positive bacterial colony into 1–2 ml of LB medium containing the appropriate antibiotic Often, the alignment of the filters with the plate does not permit identification of an individual hybridizing colony In this case, several adjacent colonies should be pooled The culture is grown for several hours and then diluted and replated on agar plates so as to obtain approximately 500 colonies per plate These colonies are then screened a second time by hybridization A single, well-isolated, positive colony should be picked from the secondary screen and used for further analysis

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2

Bacteriophage λ Vectors

Since it was first used as a cloning vehicle (Murray and Murray 1974; Rambach and Tiollais 1974; Thomas et al 1974), bacteriophage λ has occupied a central role in molecular cloning. A large number of versatile and sophisticated vectors are now available whose development was made possible by the vast and detailed analysis of the genetics and physiology of this bacteriophage The effective use of these vectors requires an understanding of the molecular biology of the virus; choosing between them demands an appreciation of the principles used in their design and construction In this chapter, we summarize the molecular biology of bacteriophage λ, describe the properties of the currently available vectors, and present detailed protocols for their growth and use

Molecular Biology of Bacteriophage λ

The genome of bacteriophage λ is a double-stranded DNA molecule approximately 50 kb in length. The DNA is carried in bacteriophage particles as a linear double-stranded molecule with single-stranded complementary termini 12 nucleotides in length (cohesive termini). Soon after entering a host bacterium, the cohesive termini associate by base pairing to form a circular molecule with two staggered nicks 12 nucleotides apart. These nicks are rapidly sealed by the host's DNA ligase to generate a closed circular DNA molecule that serves as the template for transcription during the early phase of infection During this phase, one of the following two pathways of replication is chosen (see Figure 2 1).

- During lytic growth, the circular viral DNA is replicated manyfold, a large number of bacteriophage gene products are synthesized, progeny bacteriophage particles are assembled, and the cell eventually lyses, releasing its many new infectious virus particles.
- During lysogenic growth, the infecting bacteriophage genome becomes integrated into the bacterial host DNA by site-specific recombination and is subsequently replicated and transmitted to progeny bacteria like any other chromosomal gene During lysogeny, only one bacteriophage gene is expressed· the *c*I gene, which codes for repressor.

The genes and gene products involved in each of the two pathways of bacteriophage λ growth are summarized below (For a more detailed description, see Herskowitz [1973] and Hendrix et al. [1983].)

THE LYTIC CYCLE

Adsorption

Bacteriophage λ adsorbs to receptors in the outer membrane of *Escherichia coli* that are encoded by the bacterial *lamB* gene and are normally used to transport maltose into the cell. Because the synthesis of these receptors is induced by maltose and suppressed by glucose, cultures of bacteria to be infected by bacteriophage λ should be grown in medium that contains maltose. Adsorption of bacteriophage particles to the receptors is facilitated by magnesium ions and occurs efficiently and rapidly (within a few minutes) both at room temperature and at 37°C. However, penetration of the cell by the bacteriophage DNA and the subsequent events in the lytic cycle do not occur efficiently at room temperature Plaques of bacteriophage λ will therefore not form on bacterial lawns that are incubated at room temperature.

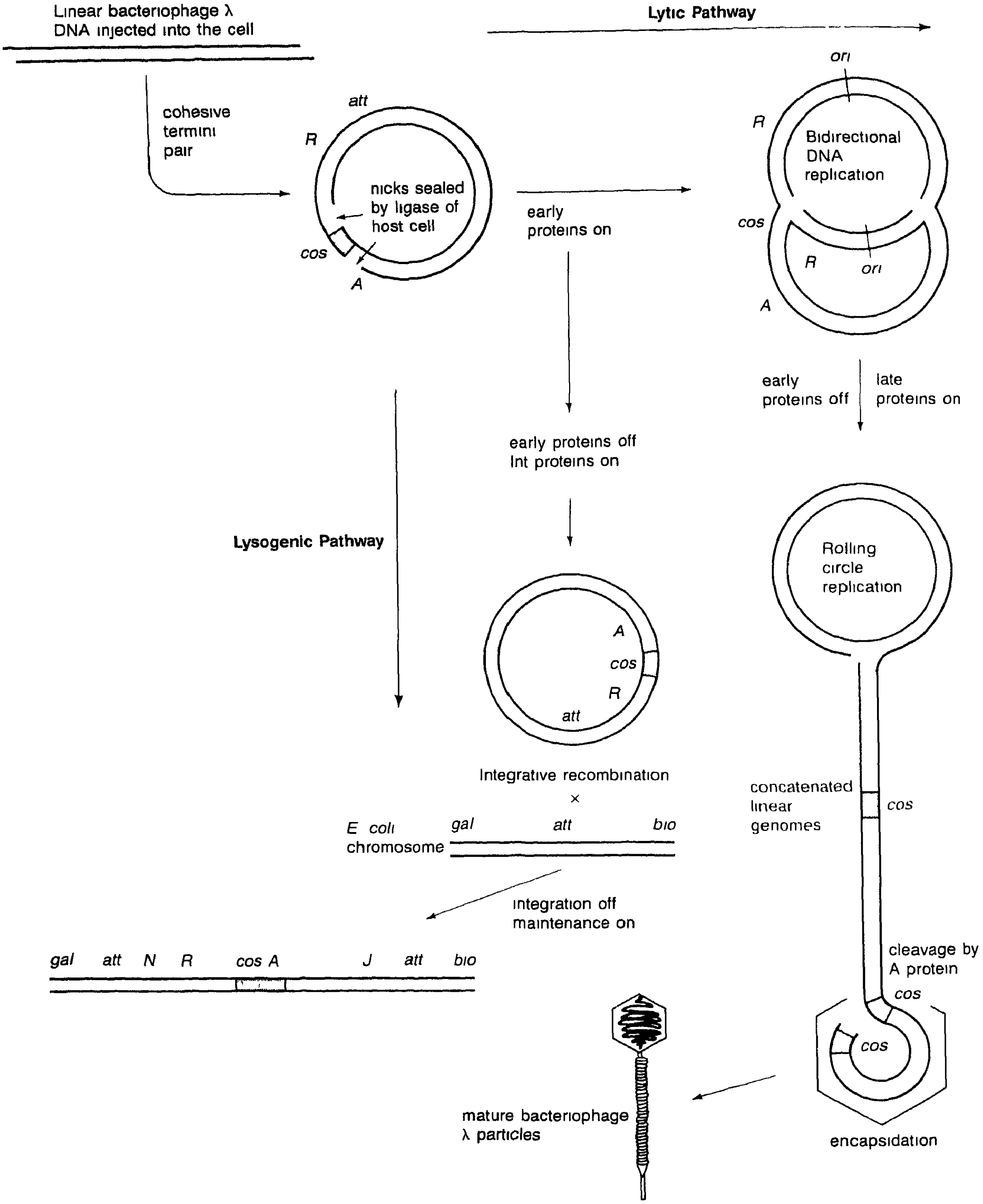


FIGURE 2.1
A schematic diagram of the bacteriophage λ life cycle Double-stranded bacteriophage λ DNA, which enters the bacterial host as a linear duplex, is indicated by double lines The lytic pathway, which is indicated by the horizontal arrow, requires early and late gene functions, whereas the lysogenic pathway, which is indicated by the vertical arrow, requires only early gene functions.

Immediate Early Transcription

Early in infection, transcription is initiated at two promoters, p_L and p_R, which are located to the left and right, respectively, of the *c*I (repressor) gene (see Figure 2 2). The resulting "immediate early" RNAs terminate at the ends of the *N* and *cro* genes, at sites t_L and t_{R1}, respectively, although some rightward transcripts continue through genes *O* and *P* (which code for proteins involved in DNA replication) and terminate at t_{R2} The leftward transcript codes for the N protein, whose action is essential for the next phase of infection

Delayed Early Transcription

The N protein, by neutralizing the activity of the host termination factor ρ, allows transcription to proceed through the early terminators t_L, t_{R1}, and t_{R2} into the remainder of the early gene region The N protein is therefore a positive regulatory element whose activity is necessary for the lytic growth of bacteriophages carrying t_{R2} However, N^- mutants can grow (albeit relatively poorly) if t_{R2} is removed by deletion Such bacteriophages are known as *nin* (*N*-*in*dependent) mutants

DNA Replication

During the early phase of infection, circular bacteriophage λ DNA replicates bidirectionally as a Cairns or θ form from a single origin (*ori*) (see Figure 2 2), which is activated by two virus-encoded proteins, the products of the *O* and *P* genes. Later, rolling-circle replication begins, and linear DNA molecules are generated that consist of tandem polymers of the bacteriophage genome arranged head-to-tail The conversion to rolling-circle replication is inhibited by exonuclease V, which is encoded by the bacterial *recB* and *recC* genes. However, the production of concatenated DNA is not affected in $recBC^+$ cells as long as the infecting bacteriophage carries a functional *gam* gene. The product of this gene binds to exonuclease V and inactivates its exonucleolytic activity During packaging, the tandem polymers are cleaved at the *cos*L and *cos*R sites by the product of the bacteriophage λ *A* gene to form the monomer-length linear DNAs with cohesive termini that are found in mature bacteriophage particles

Late Transcription

One of the early transcripts initiated at p_R codes for a protein called Cro As Cro accumulates during infection, it binds to two operator regions (o_L and o_R), preventing attachment of RNA polymerase and thereby switching off transcription from p_L and p_R. By this time, however, sufficient amounts of another control protein, called Q, have been synthesized to cause transcription of the late genes to begin. Q acts as an antiterminator of RNA synthesis from p'_R, the promoter used for transcription of the entire late region, which contains many genes involved in assembly of bacteriophage heads and tails and in cell lysis. Under the influence of Q protein, transcripts initiated at p'_R extend through the late gene region and terminate within the *b* region.

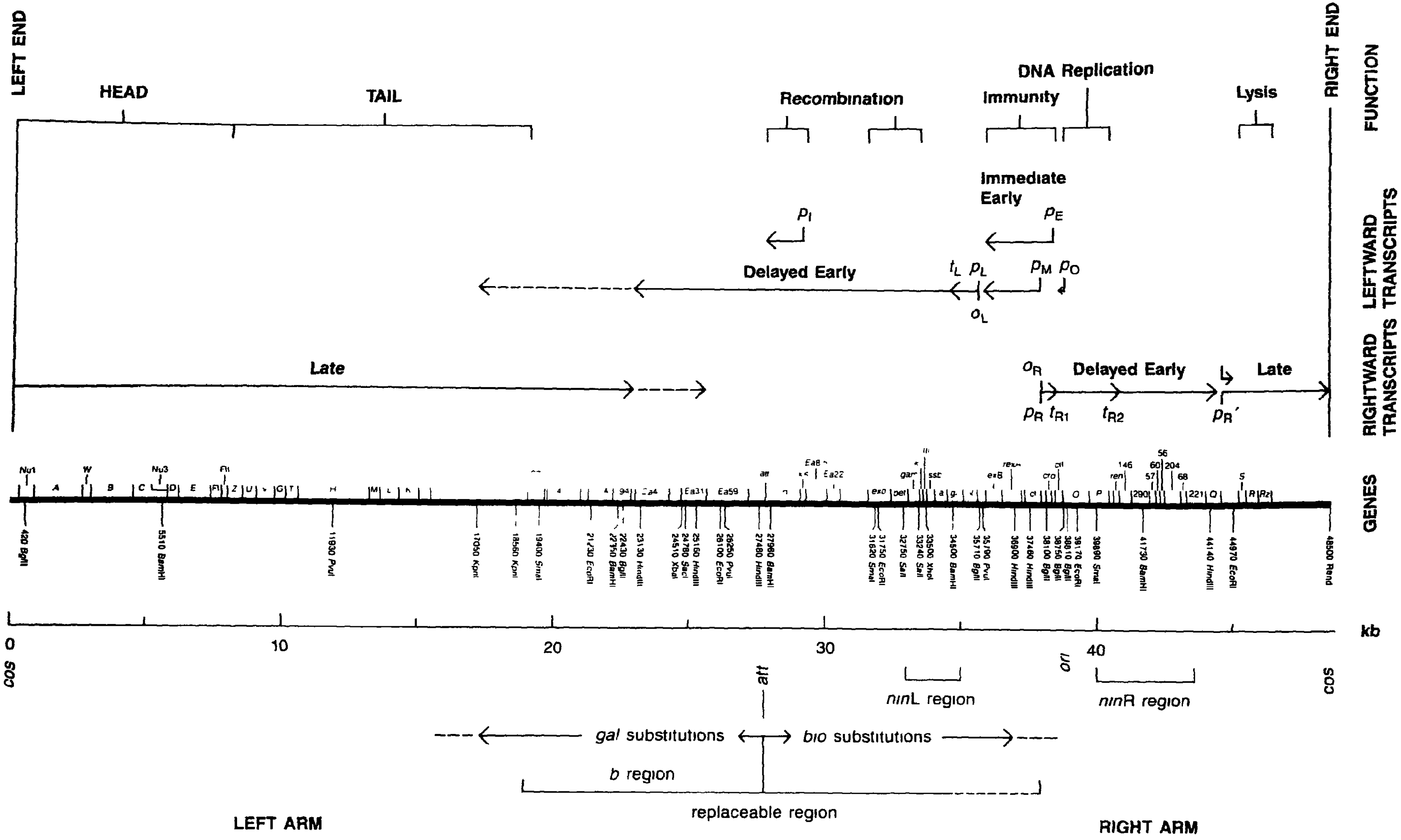


FIGURE 2.2

Physical and genetic map of wild-type bacteriophage λ. The general locations of genes that encode various lytic and lysogenic functions are indicated at the top of the figure by brackets. The genetic map below the brackets shows the specific bacteriophage λ genes (for a description of the function of each of these genes, see Hendrix et al. 1983). The locations of restriction endonuclease cleavage sites are indicated below the genetic map. Distances from the left end are given in base pairs. Leftward and rightward transcripts are shown above the genetic map. Promoters are indicated by *p*, a subscript denotes the point of origin. (p_I) *int* promoter, (p_E) establishment promoter for *c*I, (p_M) maintenance promoter for *c*I, (p_L) major leftward promoter, (p_R) major rightward promoter, (p_O) *oop* promoter, (p_R') late promoter, (→) transcription products, (——→) read through, (t_L, t_{R1}, t_{R2}) locations of *p*-mediated termination sites. Regions where substitution and deletion mutations are located are shown below the genetic map (Hendrix et al. 1983). Modified from Daniels et al. (1983).

Assembly

The two major structural units of mature bacteriophage particles—the heads and tails—are assembled separately. The earliest precursor identified in the pathway leading to head assembly is the scaffolded prehead (Figure 2.3). Further maturation, which involves removal of the scaffolding protein and proteolytic processing of other components, depends on a protein (the *groE* gene product) supplied by the host. The resulting structures are called preheads. Packaging the bacteriophage genome into preheads involves two proteins, Nu1 and A, that bind to the tandem polymers of linear DNA close to, but not at, the left *cos* site This complex then becomes attached to a defined area on the prehead. In the presence of protein FI, the DNA is wound into the prehead, which expands in size by some 20%. When the head is filled, the D, or "decoration," protein attaches to the outside of the capsid, locking the head in place around the DNA. During packaging, the left and right *cos* sites of the concatenated linear DNA are brought close together at the entrance to the head, where they are cleaved in a staggered fashion by the *ter* function of the A protein to generate the 12-nucleotide cohesive termini Finally, the filled heads associate with preformed tail units, which have been assembled by a separate pathway

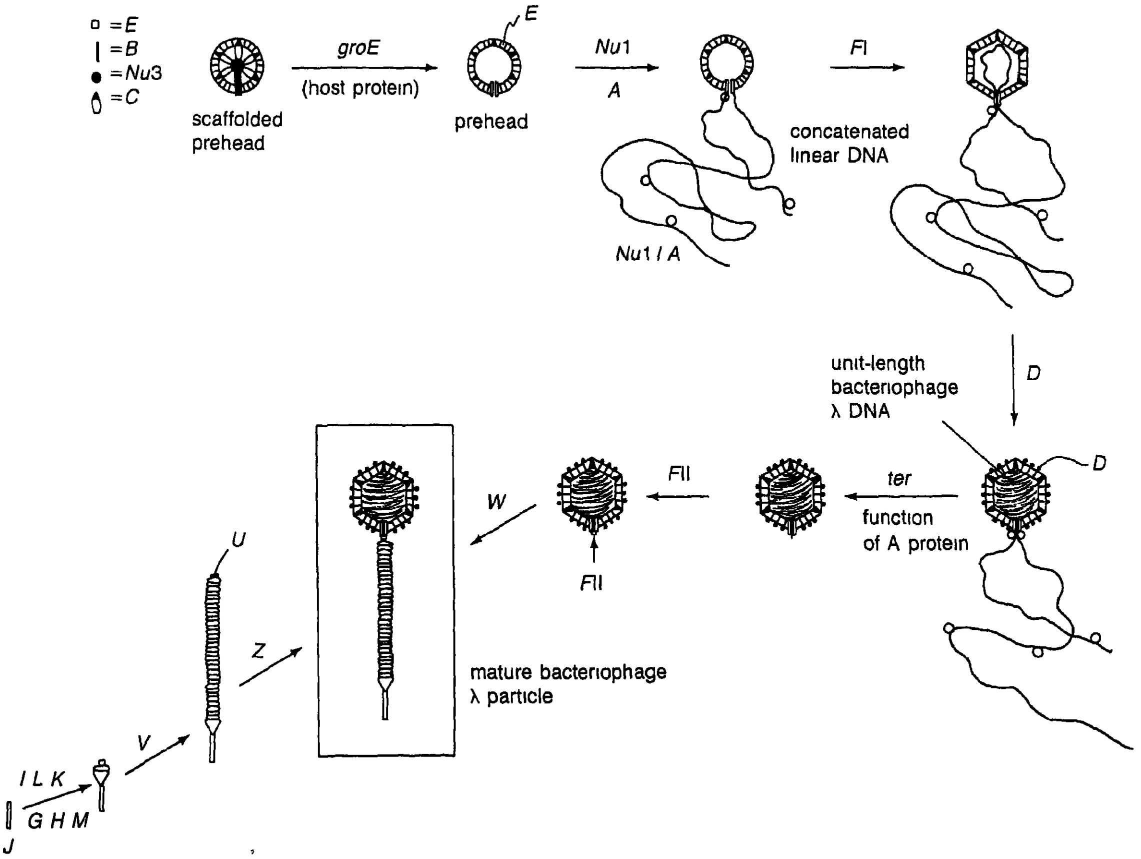


FIGURE 2.3
A schematic diagram of the assembly pathway of bacteriophage λ. The gene products involved in each step are indicated (for details, see text and Hohn 1979).

Lysis

Two bacteriophage proteins, R and S, are required for lysis of the host cell and liberation of the progeny bacteriophage Mutations in the *S* gene prevent or delay lysis, allowing the assembly of progeny particles to continue for an extended period of time. The accumulated intracellular particles are liberated by lysing the infected cell with chloroform Because of the consequent improvement in yield, most vectors in common use carry an *S* mutation

LYSOGENY

In a small proportion of cells infected by wild-type bacteriophage λ, the lytic cycle is aborted and an alternative pathway is activated that leads to the integration of viral DNA into the genome of its bacterial host The choice between the lytic and lysogenic pathways depends on an intricate balance between the activities of a number of host and bacteriophage gene products in the infected cell. To establish lysogeny, proteins encoded by genes *c*II and *c*III (whose transcriptions are controlled by p_R and p_I, respectively) activate leftward transcription from promoters p_E and p_I so that genes *c*I and *int* are expressed. The product of the *c*I gene represses early transcription and consequently blocks expression of late genes The Int protein recognizes sequences at the *att* sites in the bacterial and bacteriophage genomes and catalyzes a breaking-and-joining event that leads to insertion of the viral DNA into the host chromosome

Almost all viral transcription is repressed in the integrated state, only the *rexA, rexB,* and *c*I genes continue to be expressed The *c*I gene product, a protein of 236 amino acids, not only represses transcription of early genes but, in addition, regulates its own synthesis Low concentrations of the repressor cause the activity of the maintenance promoter, p_M, to increase, high concentrations inhibit initiation of transcription at p_M All of these effects are mediated by the binding of repressor to two operator regions (o_L and o_R) (Johnson et al 1979) Bacteriophages with mutations in gene *c*I, *c*II, or *c*III are unable to lysogenize efficiently, they therefore form clear plaques Bacteriophages carrying a temperature-sensitive mutation in the *c*I gene are able to establish and maintain the lysogenic state as long as the cells are propagated at a temperature that allows the repressor to retain its ability to inhibit transcription from p_L and p_R However, lytic growth can be induced by heating the bacterial culture briefly to 45°C to inactivate the temperature-sensitive repressor. Wild-type prophages can also be induced, although much less efficiently, by proteolytic cleavage of the repressor by the host RecA protein following exposure to agents that damage DNA Inactivation of the repressor allows transcription to resume from p_L and p_R Proteins called Int and Xis are synthesized Int and Xis together cause the *att* sites to recombine and excise the prophage DNA from the host chromosome. The lytic cycle then follows its usual course For a more detailed discussion of regulation of gene expression in bacteriophage λ, see Ptashne (1986).

Bacteriophage λ Vectors

CONSTRUCTION OF BACTERIOPHAGE λ VECTORS: A BRIEF HISTORY

At first glance, it would seem a bleak prospect to use bacteriophage λ, with its large and complex genome, as a vector. Its DNA contains multiple sites for many of the restriction enzymes that are most useful in cloning, and often these sites are located in regions of the genome essential for lytic growth of the virus. Moreover, because bacteriophage λ particles will not accommodate molecules of DNA that are much longer than the viral genome itself, it might seem that the virus would be useful as a vector for only small pieces of foreign DNA.

These problems, however, are not as formidable as they might at first appear. First, the central third of the viral genome is not essential for lytic growth. Genetic studies of specialized transducing bacteriophages, carried out many years ago (Campbell 1971), had shown that the region of the genome lying between the *J* and *N* genes could be replaced by a variety of segments of *E. coli* DNA. The detailed analysis of the structure and function of these transducing particles became a prelude to the exploration of bacteriophage λ as a vector. Without this detailed knowledge, it is certain that the virus would not have been so quickly developed as a vector, and it is quite possible that it would not have been developed at all.

Second, during the early 1970s, methods for manipulating sites of cleavage by restriction enzymes in the bacteriophage λ genome were perfected. The first of these methods, based on the knowledge of the genetics of restriction and modification of DNA by *E. coli,* allowed selection in vivo of derivatives of bacteriophage λ from which all sites for *Eco*RI had been eliminated from the essential portion of the viral genome. By growing the virus alternatively in strains of *E. coli* that either possessed or lacked a plasmid coding for *Eco*RI, mutants of bacteriophage λ were isolated that contained only one or two targets for *Eco*RI located in the nonessential region of the genome (Murray and Murray 1974; Rambach and Tiollais 1974; Thomas et al. 1974). This kind of selection in vivo was possible only when the bacteriophage could be grown in hosts that synthesized the restriction enzyme of interest, and it was necessary to develop alternative methods to obtain vectors suitable for use with other restriction enzymes. For example, recombinants between bacteriophage λ and other lambdoid bacteriophages (e.g., $\phi 80$) were constructed that either contained or lacked particular restriction sites. Later, the development of procedures for packaging purified bacteriophage λ DNA into infectious particles permitted vectors with the desired combination of restriction sites to be generated by in vitro manipulation. Bacteriophage λ DNA was digested with a restriction enzyme, and the surviving molecules were packaged into particles in vitro and propagated in *E. coli.* After several successive cycles of digestion, packaging, and growth, bacteriophage genomes were obtained that had lost restriction sites by mutation while retaining infectivity. More recently, synthetic oligonucleotides have been used to construct specifically designed DNA fragments that are then inserted into the bacteriophage λ genome Using this method, restriction sites have been placed at desired locations with an efficiency far exceeding previous methods (Frischauf et al. 1983; Karn et al 1984) Together, these methods have led to the development of a large number of vectors that can accept and propagate fragments of foreign DNA generated by a variety of different restriction enzymes (see, e g , Williams and Blattner 1980) Vectors that have a single target site for insertion of foreign DNA are known as "insertion" vectors; those that have a pair of sites flanking a segment of nonessential bacteriophage λ DNA that can be replaced by foreign DNA are known as "replacement" vectors

CHOOSING THE APPROPRIATE BACTERIOPHAGE λ VECTOR

There is no single bacteriophage λ vector suitable for cloning all DNA fragments It is therefore necessary to choose carefully among the available vectors for the one best suited to the particular task at hand. Three obvious considerations influence this choice·

- The restriction enzyme(s) to be employed
- The size of the fragment of foreign DNA to be inserted
- Whether or not the vector is to be used to express cloned DNA sequences in *E coli*

The development of vectors containing multiple cloning sites and the availability of a variety of restriction enzymes that cleave DNA to produce compatible cohesive termini have greatly simplified the mechanics of cloning in bacteriophage λ vectors However, the size of the insert remains an important factor to be considered when choosing a vector. Only about 60% of the viral genome (the left arm, ~20 kb in length, including the head and tail genes *A–J*, and the right arm, 8–10 kb in length, from p_R through the *cos*R site) is necessary for lytic growth of the bacteriophage, the central one third of the genome can be replaced by foreign DNA However, the viability of bacteriophages decreases dramatically when the lengths of their genomes are greater than 105% or less than 78% of that of wild-type bacteriophage λ. It is therefore important to choose a combination of vector and foreign DNA such that the size of the recombinant bacteriophage falls within acceptable limits These constraints can sometimes be turned to advantage After the central "stuffer" portion of some replacement vectors is removed, the deleted genome formed by fusion of the right and left arms is too short to be packaged into viable particles With such vectors, therefore, there is a positive selection for recombinant bacteriophages.

Genetic markers built into the vectors can be used to screen or select for recombinant bacteriophages (see Miller 1972) For example, vectors are available that carry a segment of *E coli* DNA coding for β-galactosidase on the stuffer fragment. Such vectors form dark blue plaques when plated on *lac*$^-$ hosts in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) Cloning into these vectors results in replacement of most of the β-galactosidase gene with foreign DNA The recombinant bacteriophages may therefore be recognized by their ability to form colorless plaques when plated on *lac*$^-$ hosts in the presence of X-gal (see Miller 1972).

Recombinant bacteriophages can be selected genetically in one of two ways. One type of genetic selection for recombinant bacteriophages can be used when cloning into the bacteriophage λ vector λgt10, which contains a single *Eco*RI site, located in the *c*I gene. This vector is *c*I$^+$ and forms lysogens with high efficiency in strains of *E. coli* carrying a mutation known as *hfl* (*h*igh *f*requency of *l*ysogenization). In *hfl* mutants, the product of the bacteriophage λ *c*II gene accumulates to much higher levels than usual *c*II is a positive regulator of the *c*I gene, so that the net effect of the *hfl* mutation is to increase the amount of the *c*I repressor in the infected cell. Lytic growth of bacteriophage λ is curtailed, and the bacteriophage genome enters the lysogenic state with very high efficiency. However, if the *cI* gene has been inactivated by insertion of foreign DNA, the growth of the bacteriophage is not restricted in *hfl* cells and the yield of progeny particles is high. This property is extremely useful when constructing recombinant DNA libraries in λgt10. A single passage of such libraries in *hfl* strains eliminates a high proportion of the nonrecombinant bacteriophages from the population and reduces significantly the labor of screening the library by hybridization.

For the second selection approach, certain vectors have been designed to take advantage of the fact that growth of wild-type bacteriophage λ is restricted in lysogens carrying prophage P2. This phenotype is called Spi$^+$ (*s*ensitive to *P*2 *i*nterference). However, strains of bacteriophage λ lacking two genes involved in recombination (*red* and *gam*) grow well in P2 lysogens and display the Spi$^-$ phenotype (Zissler et al. 1971) as long as they carry a *chi* site and the host strain is *rec*$^+$. (As discussed below, a *chi* site is a substrate for recombination catalyzed by the *recBC* system.) Several vectors, such as λ2001, λDASH, and the EMBL series (see pages 2.32–2.37), carry the *red* and/or *gam* genes in the stuffer fragment. Replacement of the stuffer fragment with a piece of foreign DNA results in recombinant bacteriophages that are Spi$^-$ and that grow efficiently in P2 lysogens of *E. coli* (Karn et al. 1980, 1984). Because recombinant bacteriophages outgrow the parental vector in these cells, it would seem to be unnecessary to take steps to minimize the number of nonrecombinants formed when constructing genomic DNA libraries—for example, by purifying the arms from the stuffer fragment. In practice, however, physical removal of the stuffer fragment has been found to improve the efficiency with which fragments of foreign DNA can be cloned and propagated in vectors of this type (Frischauf et al. 1983). The multiple restriction sites contained in the polycloning sites of the EMBL series of vectors (and in analogous vectors, λ2001 [Karn et al. 1984] and λDASH [Sorge 1988]) permit the stuffer fragment to be easily rendered unclonable by digestion with two different restriction enzymes (see page 2.92). Spi$^-$ bacteriophages show improved growth on strains of *E. coli* carrying a *recD*$^-$ mutation (Wyman et al. 1986). However, such strains do not suppress the growth of Spi$^+$ bacteriophages and therefore are not used to select Spi$^-$ recombinants.

Maintenance of the *red* and/or *gam* genes in recombinants has advantages under certain circumstances. The product of the *red* gene is involved in the recombination events early in the lytic cycle that resolve viral DNA replicating in the Cairns or θ form into closed circular progeny molecules. Later, these molecules become templates for replication by the rolling-circle mechanism, yielding linear oligomers of viral DNA, which are the preferred templates for packaging. The product of the viral *gam* gene inactivates the potent exonuclease V encoded by the host's *recB* and *recC* genes. In the absence of the *gam* gene product, the *recBC* exonuclease degrades the concatenated linear bacteriophage λ DNA produced by rolling-circle replication. Thus, in bacteria infected by *gam*$^-$ bacteriophage, the only DNA molecules available for packaging are relatively rare, exonuclease-resistant, closed circular dimers generated by θ-form replication and subsequent recombination (catalyzed by the products of the bacteriophage λ *red* gene or the host *recA* gene). Bacteriophages that are *red*$^-$ and *gam*$^-$ must therefore be propagated in *recA*$^+$ hosts in order to allow efficient packaging of their progeny DNA molecules However, the presence of an active bacterial recombination system occasionally leads to rearrangements (in particular to deletion) of segments of foreign DNA that carry repeated sequences (see, e.g , Lauer et al. 1980) To avoid this problem, several vectors have been designed that carry the *gam* gene on one of the arms of the bacteriophage DNA Examples of such vectors are λsep6-lac5 (E Meyerowitz and A. Rambach, unpubl ; see Maniatis et al. 1982 for map) and Charon 32–35 and 40 (Loenen and Blattner 1983, Dunn and Blattner 1987) Since each of these vectors is *red*$^{-}$, infected *recA* cells are phenotypically RecA^{-}, RecBC^{-}, and Red^{-}. The use of vectors like these has in some instances overcome the problems of cloning segments of foreign DNA containing recombinogenic sequences.

The final genetic factor to consider in choosing a vector is the presence of a *chi* site Wild-type bacteriophage λ contains no *chi* site, but mutants carrying an octanucleotide *chi* sequence were detected as suppressors of the small-plaque phenotype that is normally seen when *red*$^{-}$ *gam*$^{-}$ bacteriophages are plated on wild-type *E coli* Stahl and his co-workers (Stahl et al. 1975; Stahl 1979) found that *chi* sequences are preferred sites for recombination mediated by the host *recBC* enzyme The suppression of the small-plaque phenotype can therefore be explained by the observation discussed above, i.e., that *red*$^{-}$ *gam*$^{-}$ bacteriophages can package the closed circular dimers produced by θ-form replication of DNA In the presence of a *chi* sequence, the efficiency of the recombination process that gives rise to these closed circular dimers is increased and plaques are formed that are almost as large as those formed by *red*$^{+}$ *gam*$^{+}$ bacteriophages Because most bacteriophage λ vectors are *red*$^{-}$ *gam*$^{-}$ when the stuffer fragments are removed, recombinants would normally be expected to display the small-plaque phenotype. However, eukaryotic DNAs contain sequences that can mimic *chi* sites Thus, some recombinant bacteriophages will carry insertions of foreign DNA that contain *chi* sequences and others will not. The recombinants that contain *chi* sequences will grow to higher titer, form larger plaques, and become over-represented during amplification of libraries This problem can be overcome by avoiding vectors (such as Charon 28 and 30) that give rise to *gam*$^{-}$ recombinants that do not contain a *chi* site These vectors should only be used for construction of libraries if the recombinant bacteriophages are propagated on *recBC*$^{-}$ hosts For construction of genomic DNA libraries, we recommend vectors such as λ2001, λDASH, λFIX, and the EMBL series, which contain *chi* sites in the arms, or Charon 32–35 and 40, which generate *red*$^{-}$ *gam*$^{+}$ recombinants All of these vectors give rise to recombinant plaques of relatively uniform size

Recently, bacteriophage λ vectors have been developed that not only permit the propagation of foreign sequences, but also allow their expression in bacterial cells. The most commonly used vector of this sort is λgt11 (Huynh et al 1985), which carries a portion of the *E coli* β-galactosidase gene, including the upstream elements that are essential for its expression. Within the carboxy-terminal coding region of this gene is a single *Eco*RI site into which foreign DNA can be inserted λgt18–23 are derivatives of λgt11 that can accept larger inserts and that allow the use of up to four additional restriction enzymes (Han and Rutter 1987, 1988). In appropriate lytic or lysogenic hosts, the chimeric gene can be induced, resulting in the synthesis of a fusion protein consisting of the amino-terminal portion of the β-galactosidase gene fused to sequences encoded by the downstream open reading frame cDNA libraries constructed in λgt11 and λgt18–23 may be screened immunologically for expression of specific antigens in *E coli* This approach has been remarkably successful and has led to the isolation of many genes coding for proteins for which no probes other than specific antisera were available. A more recently developed bacteriophage λ expression vector, λORF8 (Meissner et al 1987), has also been used successfully (M Berman, pers comm)

A number of other genetic factors that may have a minor influence on the choice of a vector are summarized in Table 2 1, page 2 16

MAPS OF BACTERIOPHAGE λ VECTORS

Maps for each of the bacteriophage λ vectors discussed in detail in this chapter are shown on the following pages. The order of the information on each page is (from top to bottom):

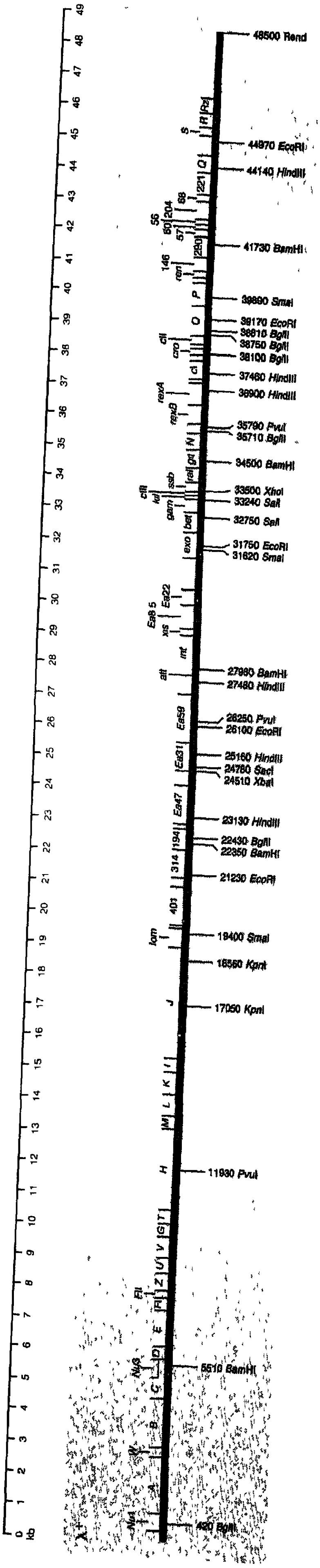
- The locations of the restriction sites and the positions of the genes of wild-type bacteriophage λ DNA
- A map of the particular vector

On the vector map, the regions derived from bacteriophage λ DNA are shown by a line whose thickness is the same as that used in the wild-type map Specific mutations (such as *W*am or *cIts*857) are indicated above the vector map. Substitutions are shown as black boxes Deletions are indicated by triangles above the map. The restriction sites present in the vector are shown below the vector map The numbers given are the best estimates available for Charon 4A and 21A; λgt10, λgt11, and λgt18–23, EMBL3, 4, and 3A; λ2001, and λORF8 The restriction site locations for Charon 32–35 and 40, λZAP, λDASH, and λFIX have been determined precisely Useful cloning sites are shown in bold. Polycloning sites are shown as "pitchforks" below the vector maps Promoters that can be used for transcription of cloned DNA (bacteriophage T3 and T7 promoters) are indicated by arrows

A description of many of the genetic markers used in bacteriophage λ vectors is presented in Table 2 1.

TABLE 2.1 Genetic Markers and Stuffer Fragments Used in Bacteriophage λ Vectors

lacZ' *lac*5 *trpE* *bio* *bio*256	Substitutions from the *lac*, *trp*, and *bio* regions of *E. coli*
*imm*80 *QSR* 80	Substitutions from bacteriophage *ϕ*80
*imm*434	Substitution from bacteriophage *ϕ*434
*imm*21	Substitution from bacteriophage *ϕ*21
*int*29	Substitution from bacteriophage *ϕ*29
*b*2 *b*1007 *b*527 *b*189	Deletions of the *b* region that damage the *att* site and therefore prevent lysogenization
KH52	A deletion in bacteriophage *ϕ*80 DNA
KH53	A deletion in the region of bacteriophage *ϕ*80, analogous to bacteriophage λ *c*I, that effectively prevents lysogenization
KH54	A deletion in the *rex c*I region that effectively prevents lysogenization
*nin*5	A deletion that removes the transcription termination site t_R and thereby renders delayed early transcription independent of the *N* gene product
*nin*L44	A deletion that removes the transcription termination site t_R, and thereby renders delayed early transcription independent of the *N* gene product and, in addition, removes the *Bam*HI site near the *ral* gene
WL113	A 1.3-kb deletion extending from the *Sal*I site at 33,240 to the *Bam*HI site at 34,500 that leaves *gam* intact and removes *kil*, *c*III, *ssb*, and *ral* (Loenen and Blattner 1983)
*ssl*Iλ1-2	A 0.5-kb deletion extending from the first *Sal*I site to the second *Sal*I site that removes *gam* and part of *bet*
*shn*dIIIλ2-3	A 2.3-kb deletion extending from the second to the third *Hin*dIII site in bacteriophage λ that removes part of the *b* region
*sx*Iλ1°	A 0.5-kb deletion created by digestion with *Xba*I followed by resection with an exonuclease (Han and Rutter 1987, 1988)
Mouse stuffer *E. coli* stuffer *lac* operator + adenovirus DNA	DNA in a vector that is replaced in the recombinants (Loenen and Blattner 1983)
Bluescript M13 -	A phagemid incorporated in λZAP that can be excised in vivo by infection with an M13 helper bacteriophage (Short et al. 1988, Stratagene Cloning Systems)
lacY lacZ lacPO *lacI*^{q1} *amp*^{r}	*lac* and *amp* incorporated in λORF8 (Meissner et al. 1987; M. Berman, pers. comm.)
*A*am *B*am *E*am *W*am *gam*am	Amber mutations that are suppressible by *supE* and/or *supF*
*int*am	An amber mutation in *int* that was created when the *Bam*HI site in *int* was destroyed
*S*am7 *S*am100	Amber mutations in a gene involved in lysis of the bacterial cell membrane. The mutations are suppressed by *supF* (but not *supE*). The absence of the wild-type *S* gene product leads to an intracellular accumulation of infectious bacteriophage particles
*c*I*ts*857	A temperature-sensitive mutation that renders the *c*I gene product thermolabile
chi	Specialized octanucleotide sites that promote directional recombination in bacteriophage λ
gam	The *gam* gene codes for an inhibitor of the *E. coli recBC* exonuclease, thereby allowing an efficient transition from θ-type DNA replication to rolling-circle replication. The *gam* gene product is important for the growth of bacteriophage λ on *recA*⁻ strains (Fec phenotype) and *recA*⁺ strains that are lysogenic for bacteriophage P2 (Spi phenotype)
exo bet (*red*)	*exo* and *bet* are the two genes located in the *red* region of bacteriophage λ (*red* is used to indicate either or both of these genes). The gene products are involved in recombination during conversion of the θ-form of replicating DNA to the rolling-circle form. They are important for growth of bacteriophage λ on *recA*⁻ strains (Fec phenotype) and on *recA*⁺ strains that are lysogenic for bacteriophage P2 (Spi phenotype)



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41730 BamHI
39890 SmaI
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38810 BglI
38750 BglI
38100 BglI
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglI
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24760 SacI
24510 XbaI
23130 HindIII
22430 BglI
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
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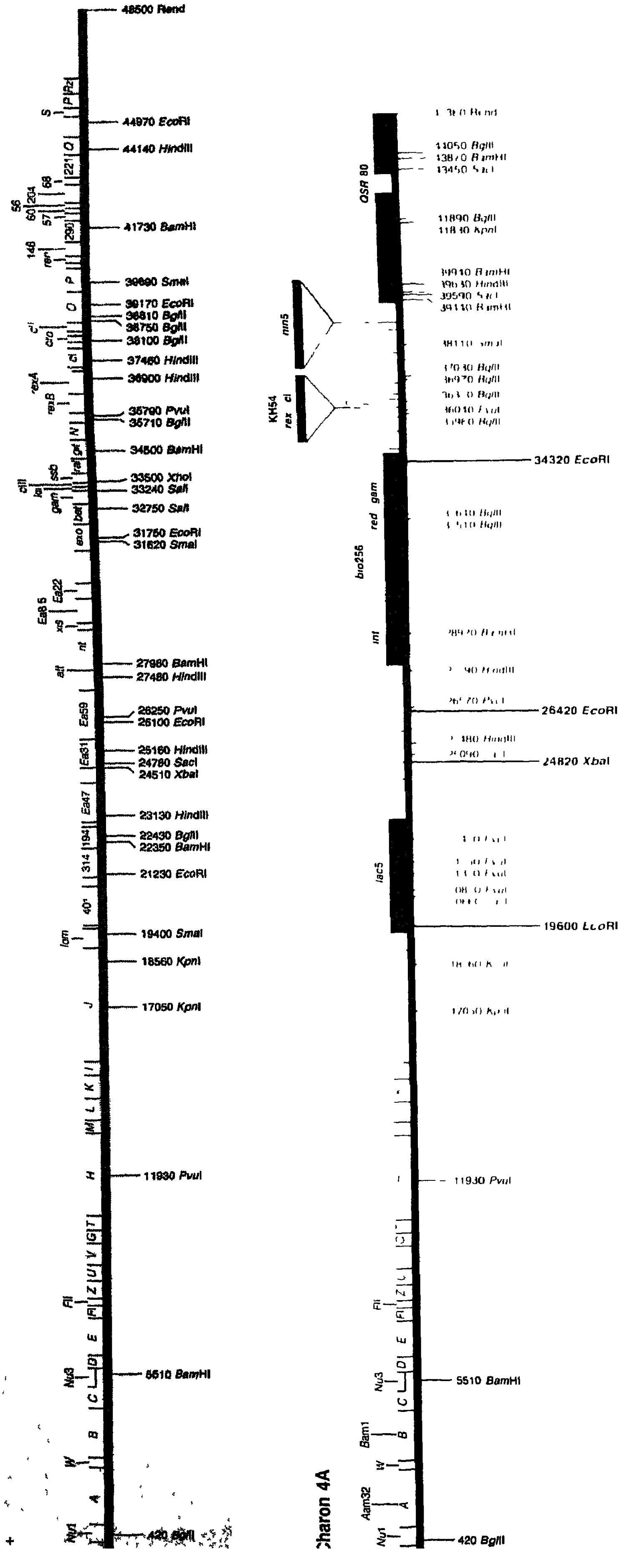
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rexB
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Ea59
Ea31
Ea47
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Nu1
QSR 80
nin5
KH54
rex
cI
red
gam
bio256
int
lac5
34320 EcoRI
26420 EcoRI
24820 XbaI
19600 EcoRI
11930 PvuI
5510 BamHI
Bam1
Aam32
420 BglII
Charon 4A

Charon 4A, 21A, 32–35, and 40

The Charon bacteriophages are replacement vectors designed for cloning large fragments of DNA The more recently constructed Charon vectors (Charon 32–35 and Charon 40) accept larger DNA fragments and have more useful restriction sites than those constructed earlier (Charon 4A and Charon 21A) Charon 40 is unique in that its stuffer consists of small DNA fragments, polymerized head-to-tail, that can be digested by a single restriction enzyme (*Nae*I) The resulting small pieces of DNA can then be removed simply and efficiently by differential precipitation with polyethylene glycol (Dunn and Blattner 1987)

Charon 32–35 and Charon 40 recombinants retain *gam* (except if the *Sal*I sites are used in Charon 32) This allows growth in *recA*$^-$ hosts and, in fact, reduces recombination to an extremely low level because the *gam* gene product inhibits the *E coli recBC* nuclease The Charon bacteriophages can be used to screen recombinant libraries by in vivo recombination (Seed 1983)

Charon 4A

Charon 4A is used chiefly as a substitution vector to construct genomic libraries of eukaryotic DNA Typically, fragments of genomic DNA, obtained by partial digestion with frequently cutting restriction enzymes and fitted with *Eco*RI linkers, are substituted for the two *Eco*RI stuffer fragments in the vector (6 9 kb and 7 8 kb) The *Eco*RI sites used for cloning are located in the *lacZ* gene (left arm) and the *bio* operon (right arm) Recombinants are therefore Lac Bio$^-$ Spi selection cannot be used for recombinants because the vector is Spi$^-$

Charon 4A contains a single *Xba*I site that can be used to clone DNA fragments up to 6 kb in length The amber mutations in genes *A* and *B* allow this vector to be used for screening recombinant libraries by in vivo recombination (Seed 1983) These amber mutations require the use of *supE* or *supF* hosts for propagation The host must also be *recA*$^+$ because the vector is *red*$^-$ *gam*

GENOTYPE

λ*A*am32 *B*am1 *lac*5 *bio*256 KH54 *srI*λ4° *nin*5 *QSR* 80

GENETIC CHARACTERISTICS

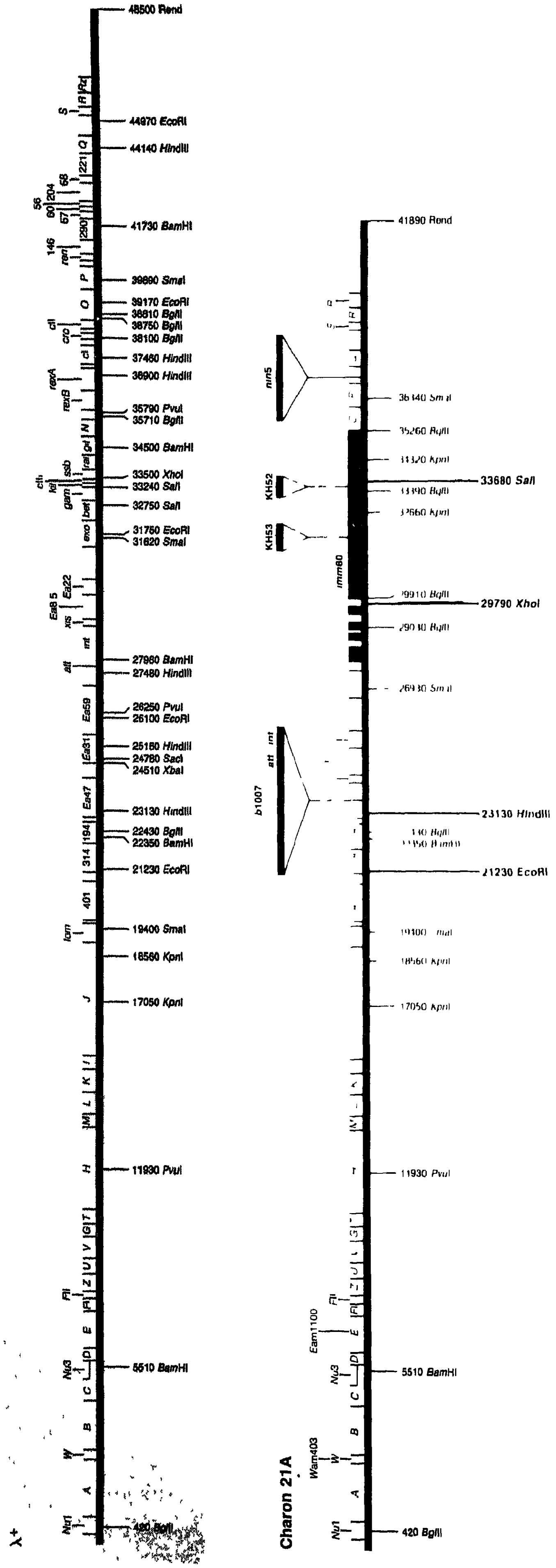
Amber mutations	*A*am32, *B*am1
Suppressor required	*supE* or *supF*
Deletions	KH54, *nin*5
Substitutions	*lac*5, *bio*256, *QSR* 80
Insertions	no
red/gam status of vector	*red*$^-$ *gam*$^-$
red/gam status of recombinants	*red gam*
chi site present in recombinants	probably
Propagates on *recA*$^-$ hosts	no
Spi selection	no
Recommended hosts	LE392

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Eco*RI	Substitution	19 5	11 0	7–20	Lac$^-$ Bio$^-$
*Xba*I	Insertion	24 8	20 5	0–6	Lac$^+$ Bio$^+$

REFERENCES

Blattner et al (1977), Williams and Blattner (1979), de Wet et al (1980)


λ+
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44970 EcoRI
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39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
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35790 PvuI
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33240 SalI
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27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
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23130 HindIII
22430 BglII
22350 BamHI
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19400 SmaI
18560 KpnI
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11930 PvuI
5510 BamHI
420 BglII
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rexB
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gam
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V
U
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
Charon 21A
41890 Rend
36140 SmaI
35260 BglII
34320 KpnI
33680 SalI
33390 BglII
32660 KpnI
29910 BglII
29790 XhoI
29040 BglII
26930 SmaI
23130 HindIII
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
nin5
KH52
KH53
imm80
b1007
att
int
Eam1100
Wam403

Charon 21A

Charon 21A is used mainly as an insertion vector to clone DNA fragments up to 9 kb in length Spi selection cannot be used because the vector is Spi$^-$ The host must carry *supE* or *supF*, because the *W* and *E* genes of the vector carry amber mutations This vector can be used to screen recombinant libraries by in vivo recombination (Seed 1983)

GENOTYPE

λWam403 *E*am1100 *b*1007 *sr*Iλ3° *imm*80 KH53 KH52 *sr*Iλ4° *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

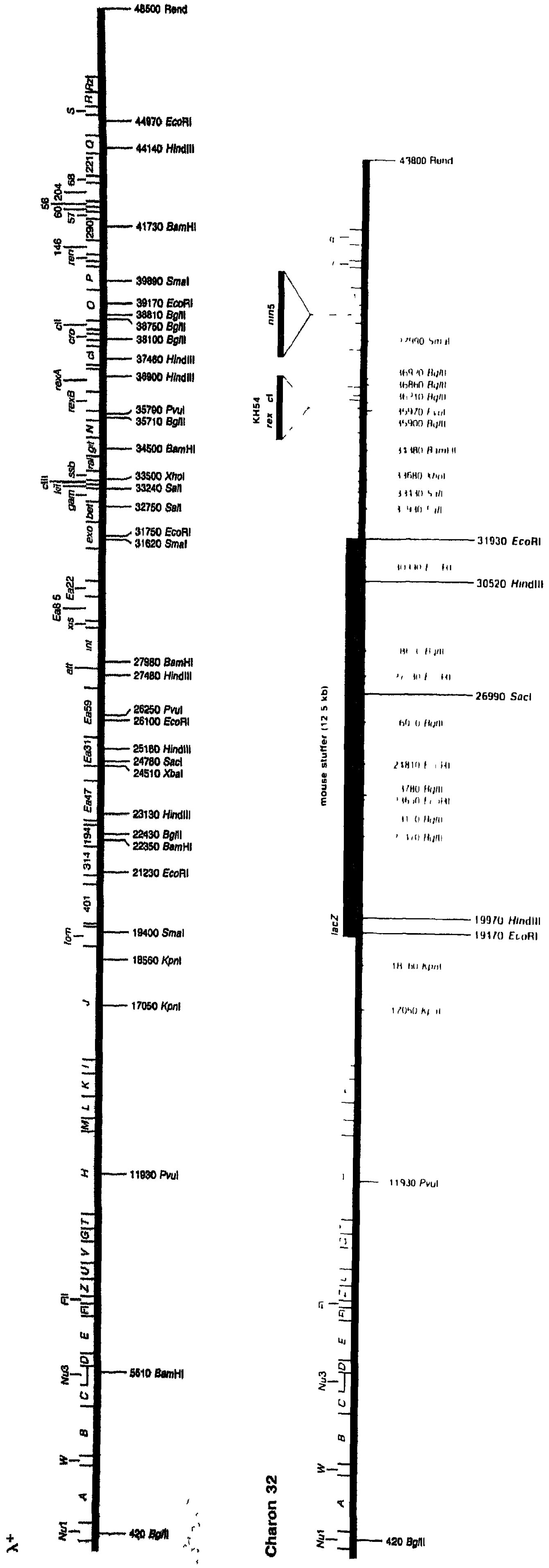
Amber mutations	*W*am403, *E*am1100
Suppressor required	*supE* or *supF*
Deletions	*b*1007, KH53, KH52, *nin*5
Substitutions	*imm*80
Insertions	no
red/*gam* status of vector	*red*$^-$ *gam*$^-$
red/*gam* status of recombinants	*red*$^-$ *gam*$^-$
chi site present in recombinants	probably
Propagates on *recA*$^-$ hosts	no
Spi selection	no
Recommended hosts	LE392

CLONING SITES

		Size (kb)		
Site	**Insertion/ substitution**	**left arm**	**right arm**	**insert**
*Eco*RI	Insertion	21 2	20 7	0–9
*Hin*dIII	Insertion	23 1	18 8	0–9
*Xho*I	Insertion	29 8	12 1	0–9
*Sal*I	Insertion	33 7	8 2	0–9
*Eco*RI + *Hin*dIII	Substitution	21 2	18 8	0–11
*Eco*RI + *Xho*I	Substitution	21 2	12 1	4 7–17 7
*Hin*dIII + *Xho*I	Substitution	23 1	12 1	2 3–15 3

REFERENCE

Williams and Blattner (1980)


λ+
Charon 32
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
S
R
Rz
Q
221
68
204
58
60
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gt
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
T
G
U
V
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
43800 Rend
nin5
KH54
rex cI
31930 EcoRI
30520 HindIII
26990 SacI
mouse stuffer (12.5 kb)
lacZ
19970 HindIII
19170 EcoRI
11930 PvuI
420 BglII

Charon 32

Charon 32 is one of several vectors designed to permit the cloning of large DNA fragments (up to 19 kb) and to allow propagation in *recA*$^-$ *E. coli*. DNA fragments with *Eco*RI ends replace a 12.5-kb stuffer fragment of mouse DNA. The left arm (19.5 kb) contains 100 bp of *lac* DNA. The right arm (11.9 kb) carries bacteriophage λ DNA from the third natural bacteriophage λ *Eco*RI site to the right end with two deletions (KH54[*rex*$^-$ *c*I$^-$] and *nin*5) and mutated *Eco*RI and *Hin*dIII sites. Charon 32 can also be used as a *Hin*dIII substitution vector or as an insertion vector to clone fragments up to 9 kb in length in the unique *Sac*I site. The vector and recombinants are able to grow in *recA*$^-$ hosts because they carry *gam*. There is no selection for recombinants.

It is also possible to use this vector as a substitution vector for *Eco*RI-*Sal*I, *Sal*I-*Xho*I, and *Eco*RI-*Bam*HI fragments. In these cases, the recombinants must be propagated on *recA*$^+$ hosts because *gam* is lost. However, Spi selection for recombinants can be used for these substitutions.

GENOTYPE

λ*sbh*Iλ1° *lac*5 *sr*I *lacZ* ⟨mouse DNA⟩ *sr*Iλ3 KH54 *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

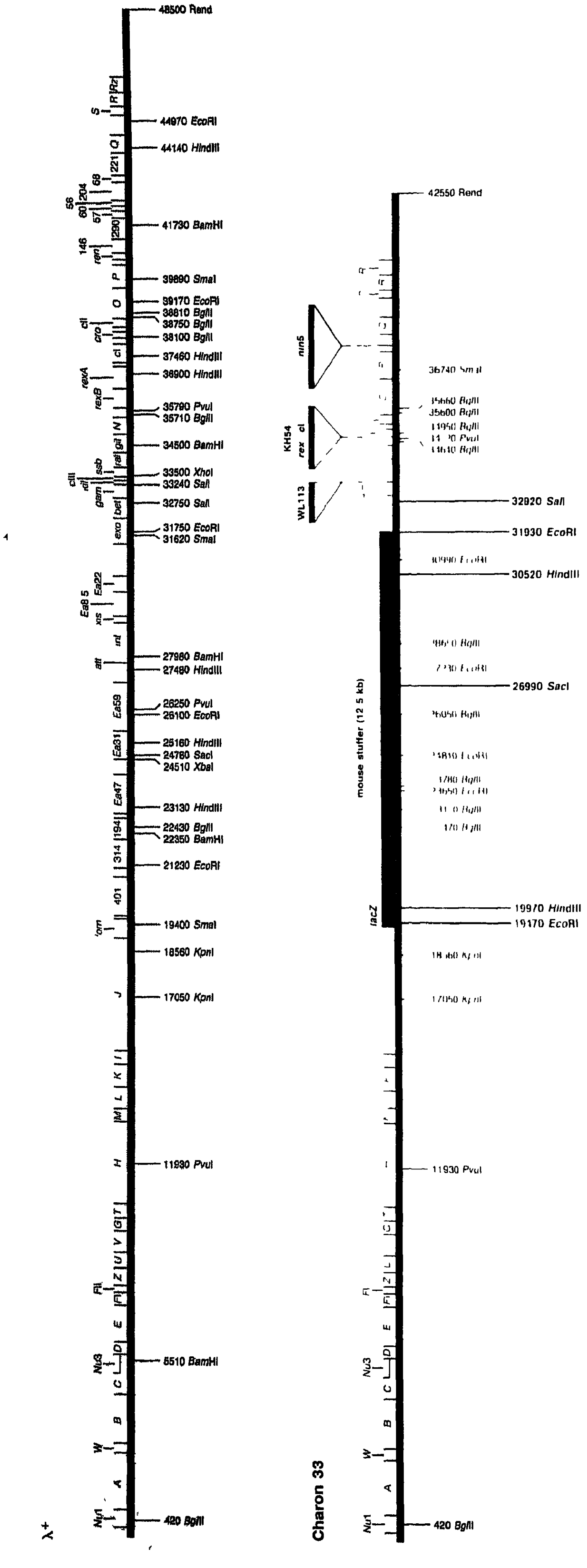
Amber mutations	no
Suppressor required	no
Deletions	KH54, *nin*5
Substitutions	12.5-kb mouse stuffer
Insertions	no
red/*gam* status of vector	*red* *gam*$^+$
red/*gam* status of recombinants	*red* *gam*$^+$
chi site present in recombinants	no
Propagates on *recA*$^-$ hosts	yes
Spi selection	no
Recommended hosts	LE392

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert
*Eco*RI	Substitution	19.5	11.9	8–19
*Hin*dIII	Substitution	20.0	13.3	5–18
*Sac*I	Insertion	27.0	16.8	0–10

REFERENCE

Loenen and Blattner (1983)

λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
S
R
Rz
Q
221
68
56
60
204
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gal
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
orf
J
I
K
L
M
H
T
G
V
U
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
Charon 33
42550 Rend
36740 SmaI
35660 BglII
35600 BglII
33950 BglII
33870 PvuI
33810 BglII
32920 SalI
31930 EcoRI
30990 EcoRI
30520 HindIII
28600 BglII
27230 EcoRI
26990 SacI
26050 BglII
24810 EcoRI
23780 BglII
23650 EcoRI
22330 BglII
21700 BglII
19970 HindIII
19170 EcoRI
18560 KpnI
17050 KpnI
11930 PvuI
420 BglII
nin5
KH54
rex
cI
WL113
mouse stuffer (12.5 kb)
lacZ
FI
Nu3
B
W
A
Nu1

Charon 33

Charon 33 is identical to Charon 32 except that the deletion WL113, which extends from bacteriophage λ *Sal*I site 2 (33,240) to bacteriophage λ *Bam*HI site 4 (34,500), has been introduced into the right arm. Thus, the left arm is 19.5 kb in length and the right arm is 10.6 kb, which allows the vector to accept *Eco*RI fragments up to 20 kb in length. Charon 33 can accept *Hin*dIII fragments up to 19 kb in length and *Sac*I inserts up to 10 kb. The deletion also allows the vector to be used as an *Eco*RI-*Sal*I vector that retains *gam* in the recombinants, which allows them to grow on *recA*$^-$ hosts.

GENOTYPE

λ*sbh*Iλ1° *lac*5 *sr*I *lacZ* ⟨mouse DNA⟩ *sr*Iλ3 ΔWL113 KH54 *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

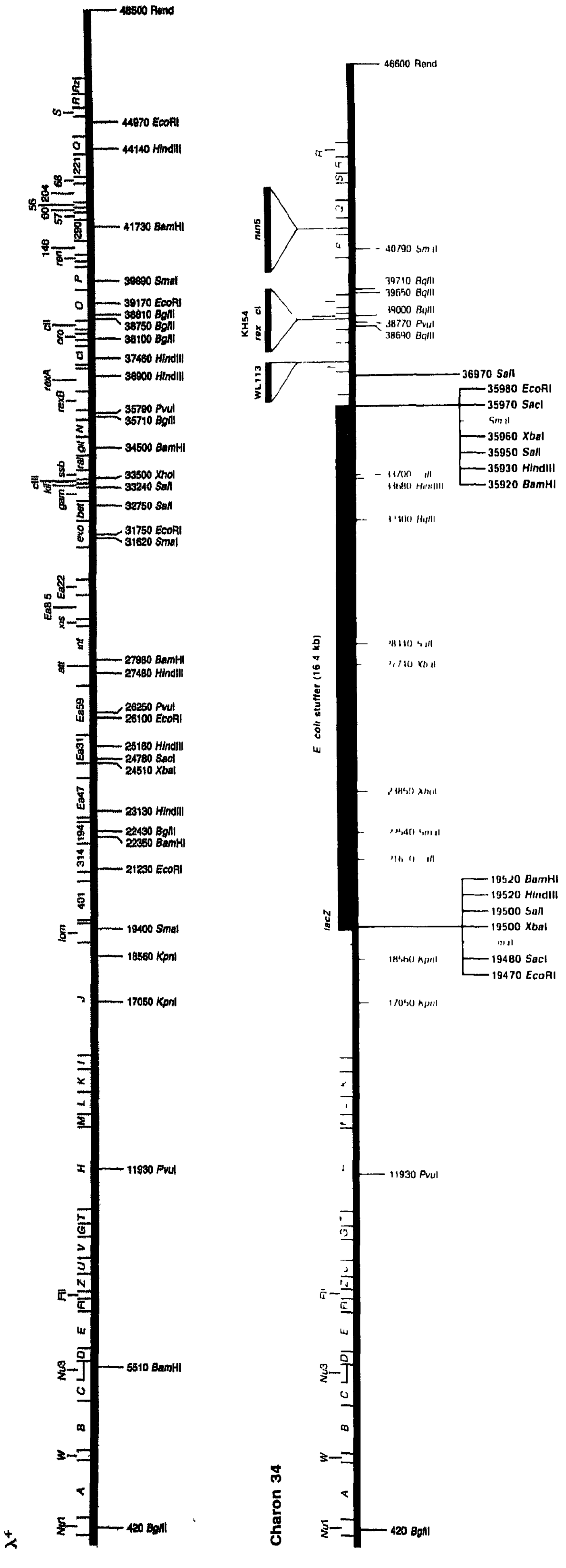
Amber mutations	no
Suppressor required	no
Deletions	WL113, KH54, *nin*5
Substitutions	12.5-kb mouse stuffer
Insertions	no
red/*gam* status of vector	*red* *gam*$^+$
red/*gam* status of recombinants	*red*$^-$ *gam*$^+$
chi site present in recombinants	no
Propagates on *recA*$^-$ hosts	yes
Spi selection	no
Recommended hosts	LE392

CLONING SITES

		Size (kb)		
Site	Insertion/ substitution	left arm	right arm	insert
*Eco*RI	Substitution	19.5	10.6	9–20
*Hin*dIII	Substitution	20.0	12.3	6–19
*Sac*I	Insertion	27.0	15.6	0–10

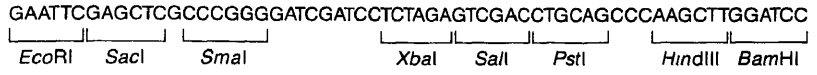
REFERENCE

Loenen and Blattner (1983)

λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25180 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
Rz
R
S
Q
221
68
204
60
56
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
cIII
kil
gam
ssb
ral
cI
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
G
T
V
U
Z
FII
E
D
Nu3
C
B
W
A
Nu1
Charon 34
46600 Rend
40790 SmaI
39710 BglII
39650 BglII
39000 BglII
38770 PvuI
38690 BglII
36970 SalI
35980 EcoRI
35970 SacI
SmaI
35960 XbaI
35950 SalI
35930 HindIII
35920 BamHI
33700
33680 HindIII
33100 BglII
28110 SalI
27730 XbaI
24850 XhoI
22540 SmaI
19520 BamHI
19520 HindIII
19500 SalI
19500 XbaI
SmaI
19480 SacI
19470 EcoRI
18560 KpnI
17050 KpnI
11930 PvuI
420 BglII
nin5
KH54
rex cI
WL113
E. coli stuffer (16.4 kb)
lacZ
R
S
Q
P
FII
E
D
Nu3
C
B
W
A
Nu1

Charon 34 and Charon 35

Charon 34 and Charon 35 allow the cloning of large DNA fragments (up to 21 kb) using any of six restriction sites located in polycloning sites in opposite orientations at the ends of stuffers. The only difference between these vectors is the source of the stuffer. To choose between these two vectors, one should consider the restriction sites within the stuffer that may influence the efficiency of separation of the arms from the stuffer fragments. All of the recombinants will retain *gam*, allowing growth on *recA*$^-$ hosts. The polycloning-site sequence is


GAATTCGAGCTCGCCCGGGGATCGATCCTCTAGAGTCGACCTGCAGCCCAAGCTTGGATCC
*Eco*RI
*Sac*I
*Sma*I
*Xba*I
*Sal*I
*Pst*I
*Hin*dIII
*Bam*HI

Charon 34

GENOTYPE

λ*sbh*Iλ1° *lac*5 *sr*I *lacZ* ⟨polycloning site–*E. coli* DNA–polycloning site⟩ *sr*Iλ3 ΔWL113 KH54 *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

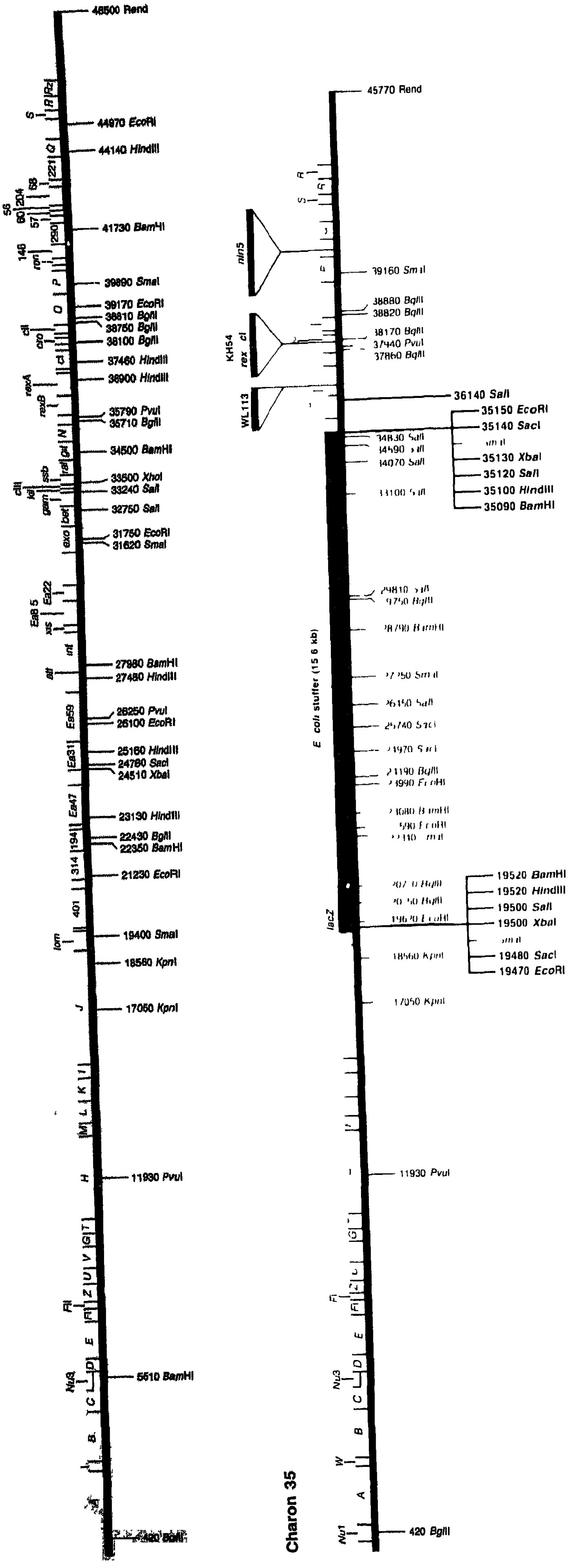
Amber mutations	no
Suppressor required	no
Deletions	WL113, KH54, *nin*5
Substitutions	16.4-kb *E. coli* stuffer
Insertions	no
red/*gam* status of vector	*red* gam^+
red/*gam* status of recombinants	*red* gam^+
chi site present in recombinants	no
Propagates on *recA* hosts	yes
Spi selection	no
Recommended hosts	LE392

CLONING SITES

Site	Insertion/ substitution	Size (kb)		
		left arm	right arm	insert
*Bam*HI *Eco*RI *Hin*dIII *Sac*I *Xba*I	Substitution	19.5	10.6	9–20
*Sal*I *Sal*I + *Eco*RI *Sal*I + *Sac*I *Sal*I + *Xba*I	Substitution	19.5	9.6	10–21

REFERENCE

Loenen and Blattner (1983)


48500 Rend
Rz
R
S
44970 EcoRI
Q
44140 HindIII
221
68
204
56
60
57
41730 BamHI
290
146
ren
39890 SmaI
P
O
39170 EcoRI
38810 BglII
38750 BglII
cII
38100 BglII
cro
cI
37460 HindIII
36900 HindIII
rexA
rexB
35790 PvuI
35710 BglII
N
34500 BamHI
gam
ssb
33500 XhoI
cIII
kil
33240 SalI
32750 SalI
bet
exo
31750 EcoRI
31620 SmaI
Ea22
Ea8.5
xis
int
27980 BamHI
27480 HindIII
att
Ea59
26250 PvuI
26100 EcoRI
Ea31
25160 HindIII
24780 SacI
24510 XbaI
Ea47
23130 HindIII
194
22430 BglII
22350 BamHI
314
21230 EcoRI
401
19400 SmaI
lom
18560 KpnI
J
17050 KpnI
I
K
L
M
H
11930 PvuI
T
G
V
U
Z
FII
FI
E
D
5610 BamHI
Nu3
C
B
420 BglII
45770 Rend
R
S
nin5
39160 SmaI
38880 BglII
38820 BglII
KH54
rex
cI
38170 BglII
37940 PvuI
37860 BglII
WL113
36140 SalI
35150 EcoRI
35140 SacI
SmaI
35130 XbaI
35120 SalI
35100 HindIII
35090 BamHI
34830 SalI
34590 SalI
34070 SalI
33100 SalI
E coli stuffer (15 6 kb)
29810 SalI
29750 BglII
28790 BamHI
27250 SmaI
26150 SalI
25740 SacI
24970 SacI
24190 BglII
23990 EcoRI
23080 BamHI
22590 EcoRI
22310 SmaI
20710 BglII
20550 BglII
19620 EcoRI
lacZ
19520 BamHI
19520 HindIII
19500 SalI
19500 XbaI
SmaI
19480 SacI
19470 EcoRI
18560 KpnI
17050 KpnI
11930 PvuI
G
U
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
420 BglII

Charon 35

Charon 35

GENOTYPE

λ*sbh*Iλ1° *lac*5 *sr*I *lacZ* ⟨polycloning site–*E coli* DNA–polycloning site⟩ *sr*Iλ3 ΔWL113 KH54 *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

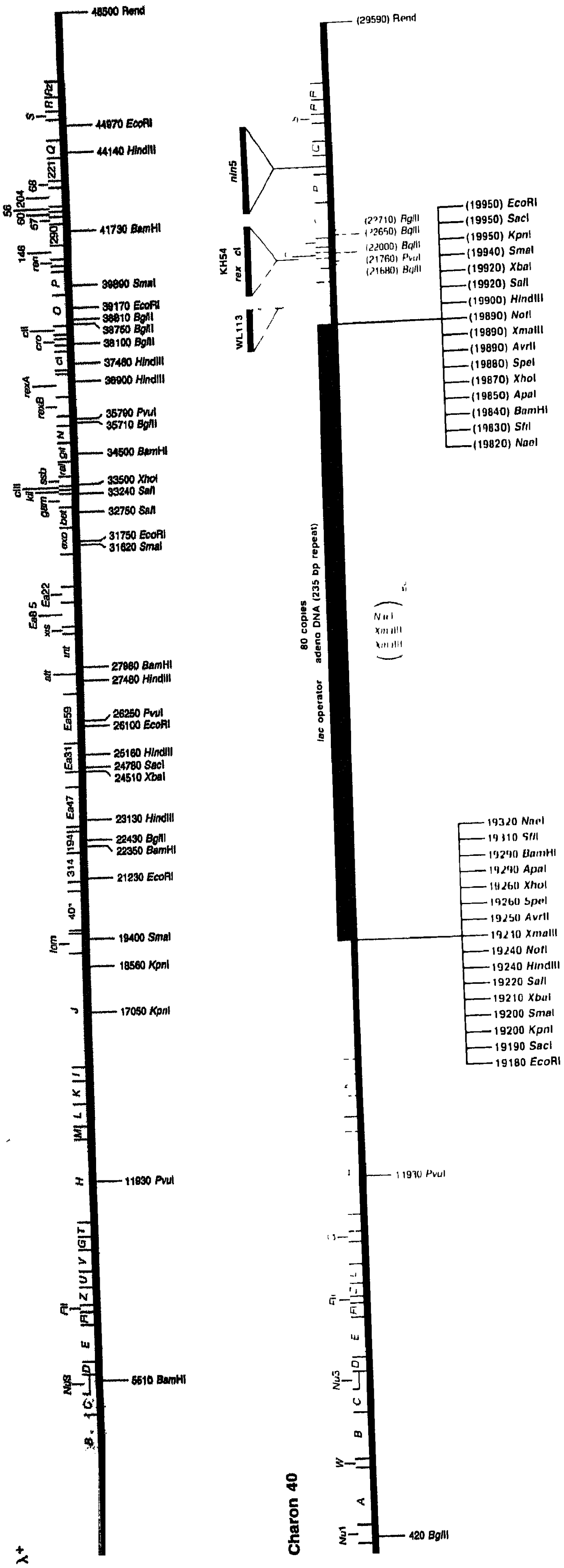
Amber mutations	no
Suppressor required	no
Deletions	WL113, KH54, *nin*5
Substitutions	15 6-kb *E coli* stuffer
Insertions	no
red/*gam* status of vector	*red* gam^+
red/*gam* status of recombinants	*red* gam^+
chi site present in recombinants	no
Propagates on *recA* hosts	yes
Spi selection	no
Recommended hosts	LE392

CLONING SITES

Site	Insertion/ substitution	Size (kb)		
		left arm	right arm	insert
*Bam*HI *Eco*RI *Hin*dIII *Sac*I *Xba*I	Substitution	19 5	10 6	9–20
*Sal*I *Sal*I + *Eco*RI *Sal*I + *Sac*I *Sal*I + *Xba*I	Substitution	19 5	9 6	10–21

REFERENCE

Loenen and Blattner (1983)


λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglI
38750 BglI
38100 BglI
37480 HindIII
36900 HindIII
35790 PvuI
35710 BglI
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglI
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
S
R
Rz
Q
221
68
204
56
60
67
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gif
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
40*
lom
J
I
K
L
M
H
T
G
V
U
Z
FII
FI
E
D
Nu3
C
B
Charon 40
(29590) Rend
nin5
KH54
rex cI
WL113
(22710) BglII
(22650) BglII
(22000) BglII
(21760) PvuI
(21680) BglII
(19950) EcoRI
(19950) SacI
(19950) KpnI
(19940) SmaI
(19920) XbaI
(19920) SalI
(19900) HindIII
(19890) NotI
(19890) XmaIII
(19890) AvrII
(19880) SpeI
(19870) XhoI
(19850) ApaI
(19840) BamHI
(19830) SfiI
(19820) NaeI
80 copies
adeno DNA (235 bp repeat)
lac operator
NotI
XmaIII
XmaIII
19320 NaeI
19310 SfiI
19290 BamHI
19290 ApaI
19260 XhoI
19260 SpeI
19250 AvrII
19210 XmaIII
19240 NotI
19240 HindIII
19220 SalI
19210 XbaI
19200 SmaI
19200 KpnI
19190 SacI
19180 EcoRI
11930 PvuI
FII
FI
E
D
Nu3
C
B
W
A
Nu1
420 BglII

Charon 40

Charon 40 is designed to accept large inserts (9 2 kb to 24 2 kb) and to provide many useful restriction sites (16) in inverted polycloning sites at opposite ends of the polystuffer The polystuffer, small DNA fragments polymerized head-to-tail, greatly facilitates removal of the stuffer from the arms The polystuffer is digested with a single restriction enzyme, *Nae*I, which cleaves the polystuffer into small pieces These small fragments can then be removed simply and efficiently by precipitation with polyethylene glycol (Dunn and Blattner 1987) Recombinants retain the *gam* gene, which allows their propagation on *recA*⁻ *E coli*

The locations of restriction sites are presented for Charon 40 carrying a two-copy polystuffer In the right polycloning site and right arm, the positions of the restriction sites are indicated in parentheses because these numbers obviously depend on the number of polystuffers present

GENOTYPE

λ*sbh*Iλ1° *sap*Iλ1° *sk*Iλ1° *sk*Iλ2° *ssm*Iλ1 ⟨polycloning site–polystuffer–polycloning site⟩ *ssl*Iλ2 ΔWL113 KH54 *ssm*Iλ3° *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

Amber mutations	no
Suppressor required	no
Deletions	WL113, KH54, *nin*5
Substitutions	polystuffer (*lac* operator + adenovirus DNA fragment) ~80×
Insertions	no
red/gam status of vector	*red*⁻ *gam*⁺
red/gam status of recombinants	*red*⁻ *gam*⁺
chi site present in recombinants	no
Propagates on *recA* hosts	yes
Spi selection	no
Recommended hosts	LE392

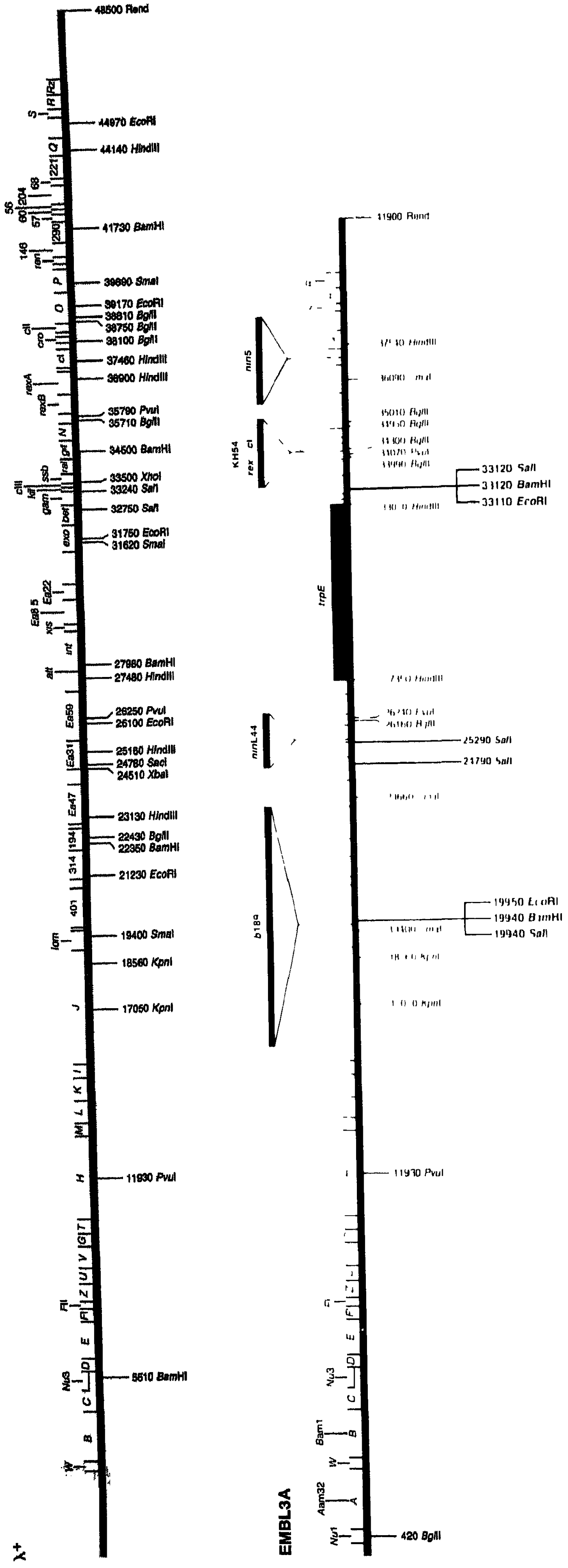
CLONING SITES

Site[a]	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Eco*RI, *Apa*I	Substitution	19 2	9 6	9 2–24 2	Most plaques are recombinants Stuffer fragments are efficiently removed
*Xba*I, *Sfi*I					
*Sac*I, *Avr*II					
*Sal*I, *Spe*I					
*Hin*dIII, *Xho*I					
*Bam*HI, *Nae*I					
*Kpn*I, *Not*I					
*Sma*I, *Xma*III					

[a] The sites are not necessarily used as the pairs listed One or two sites can be used, each site could be employed with any other site

REFERENCE

Dunn and Blattner (1987)



48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
Rz
R
S
Q
221
68
204
56
60
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gal
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
G
T
V
U
Z
FII
FI
E
D
Nu3
C
B
W
λ+
11900 Rend
33120 SalI
33120 BamHI
33110 EcoRI
25290 SalI
21790 SalI
19950 EcoRI
19940 BamHI
19940 SalI
11930 PvuI
420 BglII
nin5
KH54
cI
rex
trpE
ninL44
b189
Nu3
Bam1
Aam32
Nu1
A
EMBL3A

EMBL3, EMBL4, and EMBL3A

The EMBL vectors are replacement vectors used for cloning large (up to 20 kb) fragments of genomic DNA These vectors are particularly useful for cloning *Sau*3AI partial digests, as the *Bam*HI sites in the vectors are flanked by *Eco*RI and *Sal*I sites Cloned fragments can therefore be excised from the recombinants by digestion with *Sal*I or *Eco*RI Recombinants are Spi⁻ and thus can be selected on a P2-lysogenic host EMBL3A carries two amber mutations, allowing selection of DNA sequences linked to *supF*

The polycloning-site sequence is

```
GGATCTGGGTCGACGGATCCGGGGAATTCCCAGATCC
          L_____JL_____J  L_____J
           SalI   BamHI    EcoRI
```

In EMBL3 and EMBL3A, the polycloning sites and stuffer are as shown in the EMBL3A map *Sal*I, *Bam*HI, *Eco*RI-stuffer-*Eco*RI, *Bam*HI, *Sal*I In EMBL4, the polycloning sites are reversed *Eco*RI, *Bam*HI, *Sal*I-stuffer-*Sal*I, *Bam*HI, *Eco*RI

GENOTYPE

λ(*A*am32 *B*am1 [in EMBL3A only]) *sbh*Iλ1° *b*189 ⟨polycloning site *int*29 *nin*L44 *trpE* polycloning site⟩ KH54 *chi*C *sr*Iλ4° *nin*5 *sr*Iλ5°

GENETIC CHARACTERISTICS

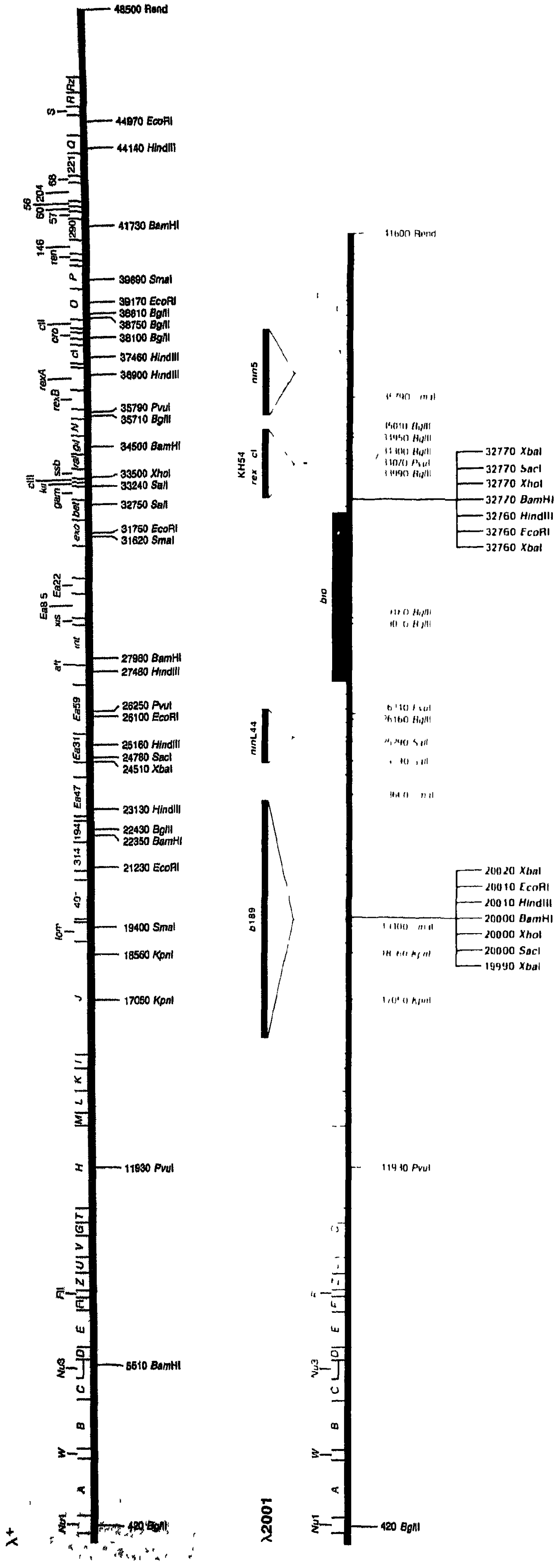
Amber mutations	no (EMBL3, EMBL4), *A*am32, *B*am1 (EMBL3A)
Suppressor required	no (EMBL3, EMBL4), *supF* (EMBL3A)
Deletions	*b*189, *nin*L44, KH54, *nin*5
Substitutions	*trpE*
Insertions	no
red/*gam* status of vector	*red*⁺ *gam*⁺
red/*gam* status of recombinants	*red*⁻ *gam*⁻
chi site present in recombinants	yes
Propagates on *recA* hosts	no
Spi selection	yes
Recommended hosts	NM538 NM539

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Sal*I *Bam*HI *Eco*RI	Substitution	19 9	8 8	7–20	Spi⁻

REFERENCE

Frischauf et al (1983)


λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
S
R
Rz
Q
221
68
56
60
204
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gal
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
40
lom
J
I
K
L
M
H
G
T
V
U
Z
FII
FI
E
Nu3
D
C
B
W
A
Nu1
λ2001
nin5
KH54
rex
cI
bio
ninL44
b189
41600 Rend
32770 XbaI
32770 SacI
32770 XhoI
32770 BamHI
32760 HindIII
32760 EcoRI
32760 XbaI
20020 XbaI
20010 EcoRI
20010 HindIII
20000 BamHI
20000 XhoI
20000 SacI
19990 XbaI
11930 PvuI
420 BglII

2001, λDASH, and λFIX

λ2001, λDASH, and λFIX contain multiple cloning sites in polycloning-site sequences that are inverted with respect to each other Large DNA fragments can be cloned in these replacement vectors. Inserts that destroy the *Xho*I or *Bam*HI sites can be excised at flanking sites The right arm of each carries a *chi*C mutation that facilitates growth The *red gam* region is lost in the recombinants, so Spi selection can be used in hosts lysogenic for bacteriophage P2

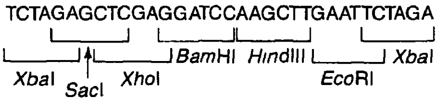
λDASH and λFIX are identical, apart from their polycloning sites These bacteriophages have bacteriophage T7 and T3 promoters in the polycloning sites. Thus, RNA probes can be synthesized without subcloning, and chromosome walking is made easier

λ2001

λ2001 contains multiple cloning sites (*Xba*I, *Sac*I, *Xho*I, *Bam*HI, *Hin*dIII, and *Eco*RI) and is used to clone large (10 kb to 22 kb) DNA fragments The polycloning-site sequences are inverted with respect to each other Inserts that destroy the *Xho*I or *Bam*HI site can be excised at flanking sites *Eco*RI and *Hin*dIII sites in the right arm of bacteriophage λ have been mutated The right arm carries a *chi*C mutation that facilitates growth on *rec*$^+$ hosts

Spi$^-$ selection can be used because the *red gam* region is lost in the recombinants A host lysogenic for bacteriophage P2 is used to select for recombinants

The polycloning-site sequence is



GENOTYPE

λ*sbh*Iλ1° *b*189 ⟨polycloning site *sr*Iλ3° *nin*L44 *bio* polycloning site⟩ KH54 *chi*C *sr*Iλ4° *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

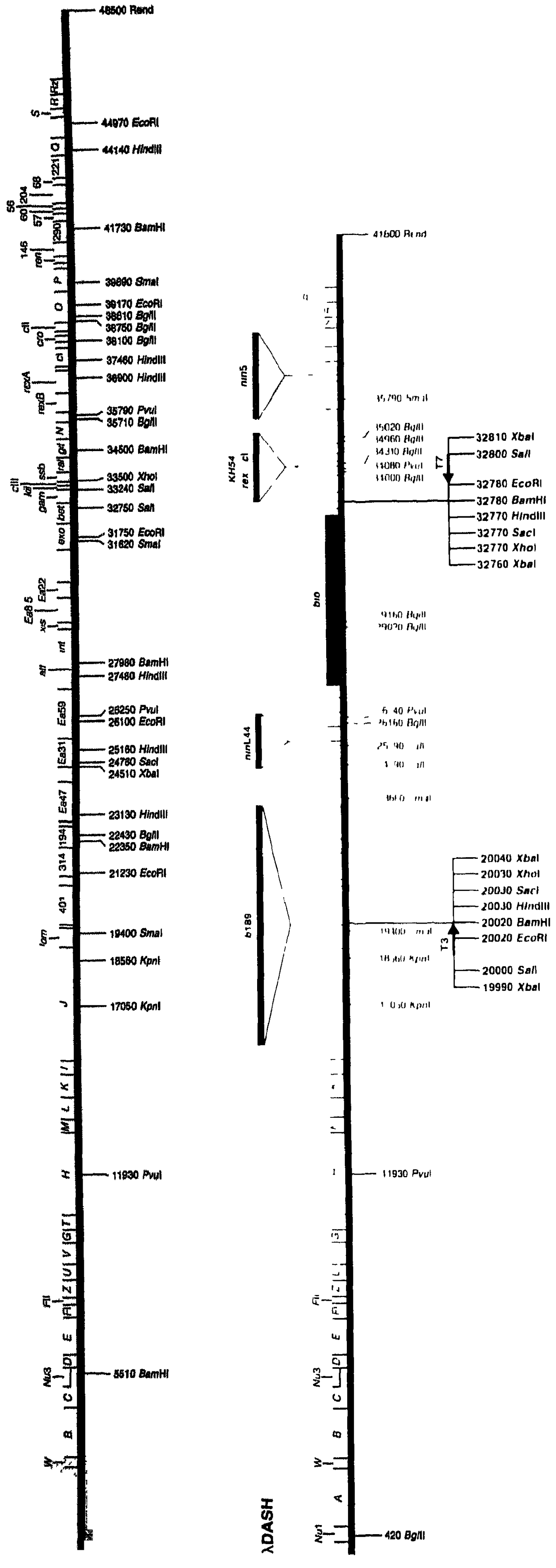
Amber mutations	no
Suppressor required	no
Deletions	*b*189, *nin*L44, KH54, *nin*5
Substitutions	*bio*
Insertions	no
red/gam status of vector	*red*$^+$ *gam*$^+$
red/gam status of recombinants	*red*$^-$ *gam*$^-$
chi site present in recombinants	yes
Propagates on *recA*$^-$ hosts	no
Spi selection	yes
Recommended hosts	NM539

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Xba*I *Sac*I *Xho*I *Bam*HI *Hin*dIII *Eco*RI	Substitution	20	9	10–22	Spi$^-$

REFERENCE

Karn et al (1984)



48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18580 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
S
R
Rz
Q
221
68
204
56
60
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gif
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
T
G
V
U
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
λDASH
41600 Rend
nin5
KH54
rex cI
bio
ninL44
b189
T7
T3
32810 XbaI
32800 SalI
32780 EcoRI
32780 BamHI
32770 HindIII
32770 SacI
32770 XhoI
32760 XbaI
20040 XbaI
20030 XhoI
20030 SacI
20030 HindIII
20020 BamHI
20020 EcoRI
20000 SalI
19990 XbaI
11930 PvuI
420 BglII

λDASH and λFIX

λDASH contains multiple cloning sites (*Xba*I, *Sal*I, *Eco*RI, *Bam*HI, *Hin*dIII, *Sac*I, and *Xho*I) and is used to clone large (9 kb to 22 kb) DNA fragments The polycloning-site sequences are inverted with respect to one another Inserts that destroy the *Xho*I or *Bam*HI site can be excised at flanking sites The bacteriophage T3 and T7 promoters adjacent to the polycloning sites allow RNA probes to be made without subcloning This simplifies chromosome walking, since subcloning into plasmids is not necessary

*Eco*RI and *Hin*dIII sites in the right arm of bacteriophage λ have been mutated The right arm carries a *chi*C mutation that facilitates growth on rec^+ hosts Spi$^-$ selection can be used because the *red gam* region is lost in the recombinants A bacterial host lysogenic for bacteriophage P2 is used to select for recombinants

λFIX is similar to λDASH but has different polycloning sites *Xba*I, *Sac*I, bacteriophage T7 promoter, *Sal*I, *Xho*I, *Eco*RI, *Xba*I–stuffer–*Xba*I, *Eco*RI, *Xho*I, *Sal*I, bacteriophage T3 promoter, *Sac*I, *Xba*I

λDASH

GENOTYPE

λ*sbh*Iλ1° *b*189 ⟨T3 promoter–polycloning site *sr*Iλ3° *nin*L44 *bio* polycloning site–T7 promoter⟩ KH54 *chi*C *sr*Iλ4° *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

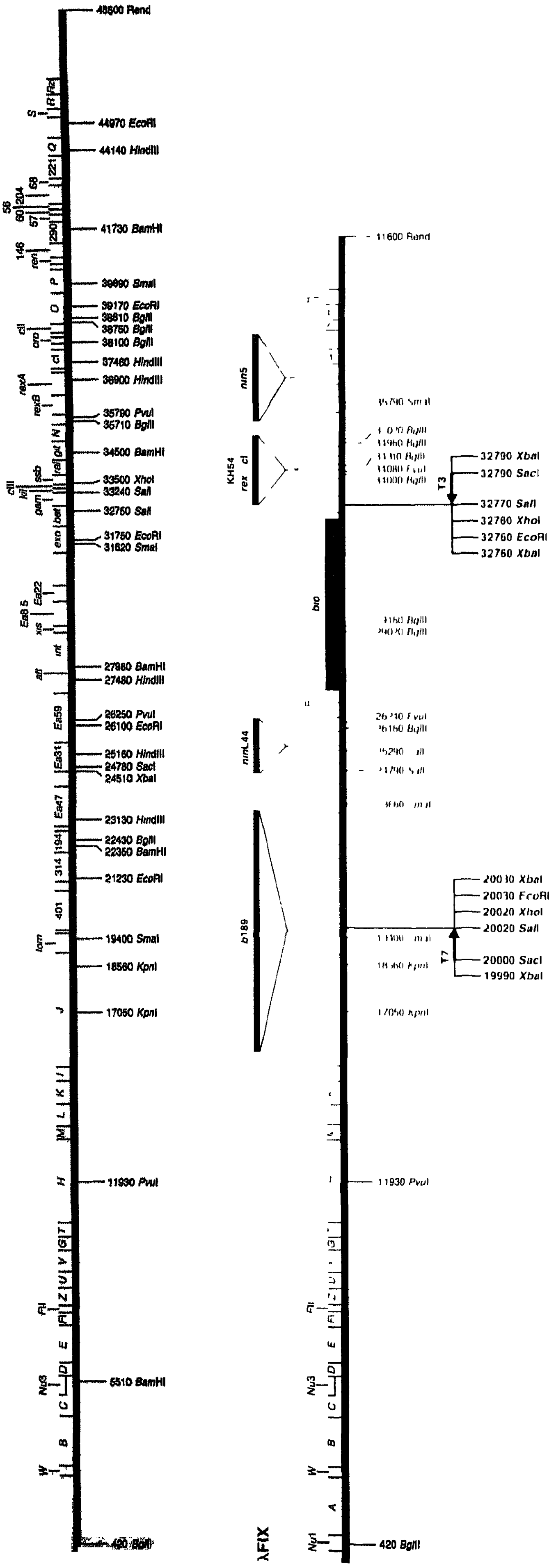
Amber mutations	no
Suppressor required	no
Deletions	*b*189, *nin*L44, KH54, *nin*5
Substitutions	*bio*
Insertions	bacteriophage T3 promoter in left polycloning site bacteriophage T7 promoter in right polycloning site
red/*gam* status of vector	red^+ gam^+
red/*gam* status of recombinants	red^- gam^-
chi site present in recombinants	yes
Propagates on $recA^-$ hosts	no
Spi selection	yes
Recommended hosts	NM539

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Xba*I *Sal*I *Eco*RI *Bam*HI *Hin*dIII *Sac*I *Xho*I	Substitution	20	9	9–22	Spi$^-$

REFERENCES

Sorge (1988), Stratagene Cloning Systems


48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
S
R
Rz
Q
221
68
56
204
60
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gif
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
T
G
V
U
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
nin5
KH54
rex
cI
ninL44
b189
λFIX
bio
11600 Rand
11930 PvuI
420 BglII
17050 KpnI
T3
32790 XbaI
32790 SacI
32770 SalI
32760 XhoI
32760 EcoRI
32760 XbaI
T7
20030 XbaI
20030 EcoRI
20020 XhoI
20020 SalI
20000 SacI
19990 XbaI

λFIX

GENOTYPE

λ*sbh*Iλ1° *b*189 ⟨T7 promoter–polycloning site *sr*Iλ3° *nin*L44 *bio* polycloning site–T3 promoter⟩ KH54 *chi*C *sr*Iλ4° *nin*5 *shnd*IIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

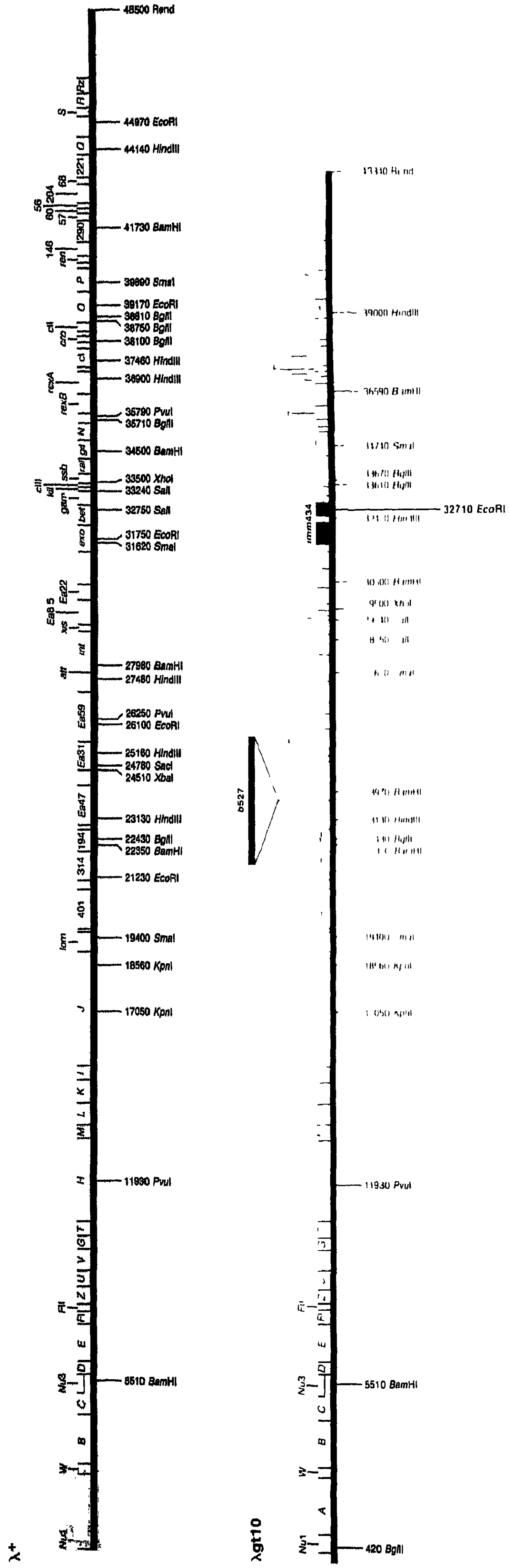
Amber mutations	no
Suppressor required	no
Deletions	*b*189, *nin*L44, KH54, *nin*5
Substitutions	*bio*
Insertions	bacteriophage T7 promoter in left polycloning site bacteriophage T3 promoter in right polycloning site
red/*gam* status of vector	*red*$^+$ *gam*$^+$
red/*gam* status of recombinants	*red*$^-$ *gam*$^-$
chi site present in recombinants	yes
Propagates on *recA*$^-$ hosts	no
Spi selection	yes
Recommended hosts	NM539

CLONING SITES

		Size (kb)			
Site	**Insertion/ substitution**	**left arm**	**right arm**	**insert**	**Recombinants**
*Xba*I *Sac*I *Sal*I *Xho*I *Eco*RI	Substitution	20	9	9–22	Spi$^-$

REFERENCE

Stratagene Cloning Systems


λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
S
R
Rz
Q
221
68
56
60
204
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gd
ssb
kil
cIII
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
T
G
V
U
Z
FII
FI
E
D
Nu3
C
B
W
Nu1
λgt10
39000 HindIII
36590 BamHI
32710 EcoRI
imm434
b527
11930 PvuI
5510 BamHI
420 BglII
FI
E
D
Nu3
C
B
W
A
Nu1

λgt10 is designed for cloning small (~6 kb) cDNA fragments into the unique *Eco*RI site in the immunity region This vector is often used when the amount of foreign DNA is limited Cloning efficiency is high, and recombinants can be selected on an *E coli* strain carrying the *hflA*150 mutation because the recombinants are *c*I$^-$ *c*I$^+$ bacteriophages lysogenize very efficiently on an *hflA* strain, and plaque formation is suppressed *c*I$^-$ recombinants grow normally on this strain Recombinants retain *red* and *gam,* which allows propagation on *recA*$^-$ hosts

GENOTYPE

λ*srI*λ1° *b*527 *srI*λ3° *imm*434 (*srI*434$^+$) *srI*λ4° *srI*λ5°

GENETIC CHARACTERISTICS

Amber mutations	no
Suppressor required	no
Deletions	*b*527
Substitutions	*imm*434
Insertions	no
red/gam status of vector	*red*$^+$ *gam*$^+$
red/gam status of recombinants	*red*$^+$ *gam*$^+$
chi site present in recombinants	no
Propagates on *recA*$^-$ hosts	yes
Spi selection	no
Recommended hosts	C600 (BNN93) to propagate vector BNN102 (C600*hflA*) to screen for recombinants

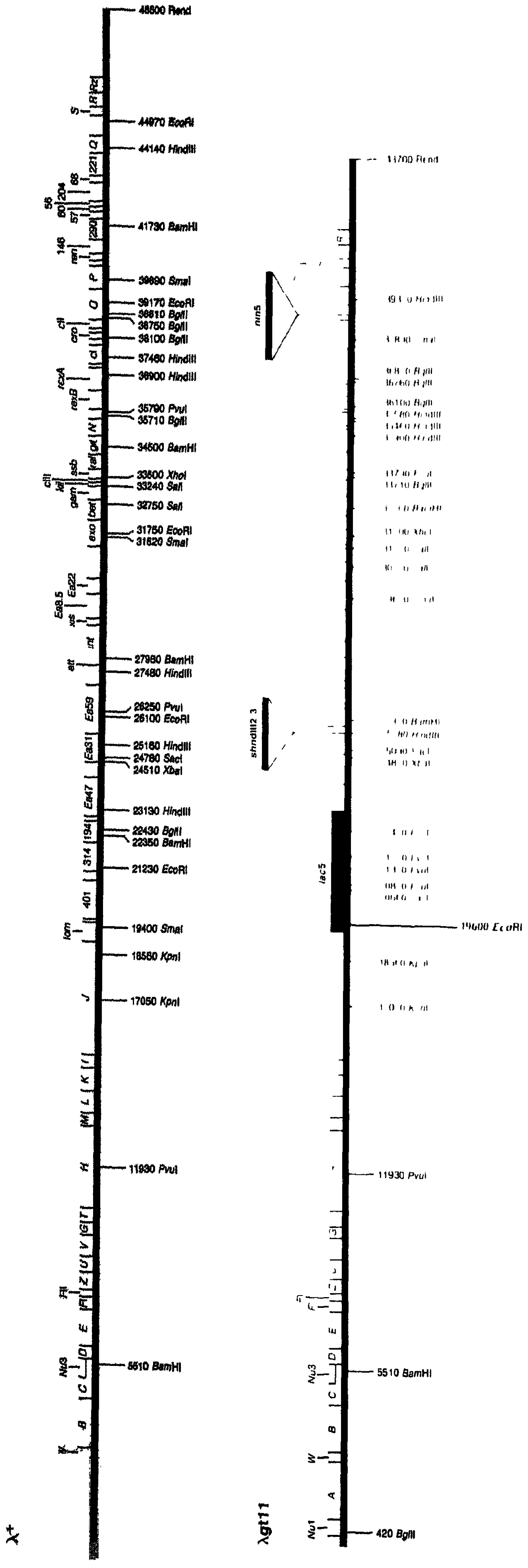
CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert[a]	Recombinants
*Eco*RI	Insertion	32 7	10 6	0–6	*c*I$^-$

[a] The maximum theoretical size of cDNA that can be propagated in λgt10 is ~7 6 kb However, to allow a margin of safety, cDNA estimated to be larger than ~6 kb should be cloned in a vector with a greater capacity (Kaiser and Murray 1985)

REFERENCE

Huynh et al (1985)


λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24760 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
Rz
R
S
Q
221
68
204
56
60
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290
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ren
P
O
cII
cro
cI
rexA
rexB
N
gol
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
T
G
V
U
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
λgt11
43700 Rend
nin5
shndIII2 3
lac5
19600 EcoRI
11930 PvuI
5510 BamHI
420 BglII

t11 and λgt18–23

This λgt series of insertion vectors is designed to express cDNA as a β-galactosidase fusion protein. They are often used for immunological screening of either plaques or colonies. Recombinants are Lac$^-$ because the insertion sites are in *lacZ*. The *S*am100 mutation allows propagation and screening in suppressing hosts and intracellular accumulation of the fusion protein in nonsuppressing *E. coli*. The temperature-sensitive repressor can be used to control bacteriophage replication and production of fusion protein.

To construct λgt18 and λgt19, the two bacteriophage λ *Sal*I sites were destroyed and a polycloning site containing a *Sal*I site was introduced at the *Eco*RI site at the 3′ terminus of *lacZ*. The polycloning-site sequences are shown on page 2.45.

To construct λgt20 and λgt21, a *Sac*I and *Xba*I site were eliminated. In addition, a synthetic *chi* site was inserted and a small region in the bacteriophage λ sequences adjacent to *lacZ* was deleted. In λgt22 and λgt23, a *Not*I site was added.

In these bacteriophages, cDNA can be cloned in a defined orientation. In addition, the use of *Not*I or *Sal*I linkers eliminates the need to methylate cDNA. Because *Not*I and *Sal*I sites occur very infrequently in mammalian DNA, there is little chance that a cDNA clone will be cleaved by these enzymes.

λgt11

λgt11 is a vector commonly used for immunological screening. DNA fragments (up to 7.2 kb) are cloned into the unique *Eco*RI site located in *lacZ*, allowing expression of a fusion protein if the cloned sequence is in-frame with *lacZ*. A *supF* host is used for propagation and screening. In a nonsuppressing host, the Sam100 mutation prevents cell lysis and allows intracellular accumulation of the desired fusion protein. The bacteriophage carries *red* and *gam* and thus can be grown in a *recA*$^-$ host. The temperature-sensitive repressor allows control of bacteriophage replication and production of fusion proteins.

GENOTYPE

λ*lac*5 Δ*shn*dIIIλ2-3 *sr*Iλ3° *c*I*ts*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

GENETIC CHARACTERISTICS

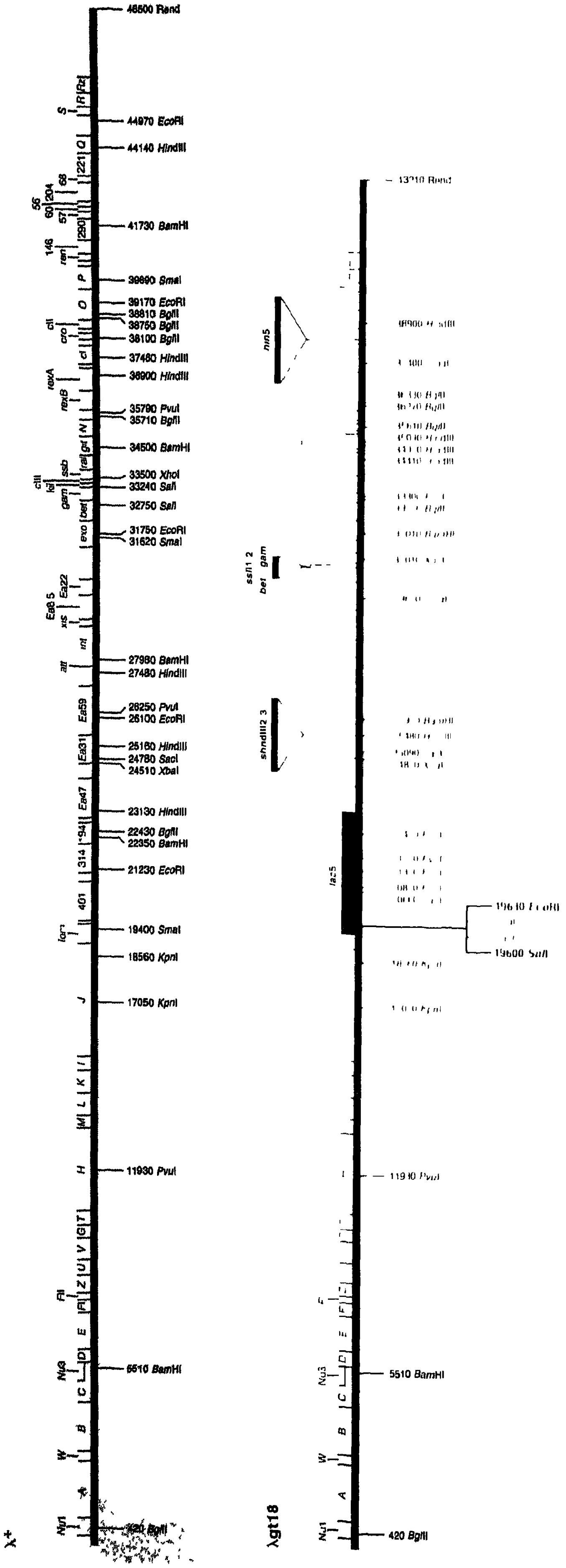
Amber mutations	*S*am100
Suppressor required	*supF*
Deletions	*shn*dIIIλ2-3, *nin*5
Substitutions	*lac*5
Insertions	no
red/*gam* status of vector	*red*$^+$ *gam*$^+$
red/*gam* status of recombinants	*red*$^+$ *gam*$^+$
chi site present in recombinants	no
Propagates on *recA*$^-$ hosts	yes
Spi selection	no
Recommended hosts	Y1090*hsdR*

CLONING SITES

Site	Insertion/ substitution	Size (kb)			Recombinants
		left arm	right arm	insert	
*Eco*RI	Insertion	19.5	24.2	0–7.2	Lac$^-$

REFERENCE

Young and Davis (1983a)


λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37480 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
Rz
R
S
Q
221
68
204
56
60
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
gal
ral
ssb
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
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I
K
L
M
H
G
T
V
U
Z
FII
FI
E
Nu3
D
C
B
W
A
Nu1
λgt18
nin5
ssn1 2
bet gam
shndIII2 3
lac5
19610 EcoRI
19600 SalI
11930 PvuI
5510 BamHI
420 BglII
Nu3
D
C
B
W
A
Nu1
F
E

λgt18 and λgt19

λgt18 and λgt19 are derivatives of λgt11 and are used to clone cDNA fragments into unique *Eco*RI or *Sal*I restriction sites within *lacZ*. Thus, as with λgt11, the two vectors can be used for immunological screening. The two natural bacteriophage λ *Sal*I sites were destroyed by digesting λgt11 with *Sal*I and then ligating the bacteriophage λ arms in the presence of an oligonucleotide that does not reconstruct the *Sal*I sites. This alteration causes the vector to become *red*$^-$ *gam*$^-$. The resulting small-plaque phenotype was altered by passing the vector through five generations of growth and picking a large plaque. Thus, the resulting vector is Spi$^-$ and probably *chi*$^+$. A polycloning site with *Sal*I, *Sac*I, *Xba*I, and *Eco*RI sites was inserted at the *Eco*RI site in *lacZ*. Only *Sal*I and *Eco*RI sites are unique in these vectors. The only difference between λgt18 and λgt19 is the orientation of the polycloning site. The DNA and amino acid sequences introduced are

	Glu	Phe	Ser	Arg	Glu	Leu	Val	Asp	Gln	Phe
λgt18	GAA	TTC	TCT	AGA	GAG	CTC	GTC	GAC	CAA	TTC
	*Eco*RI		*Xba*I		*Sac*I		*Sal*I			

	Glu	Leu	Val	Asp	Glu	Leu	Ser	Arg	Glu	Phe
λgt19	GAA	TTG	GTC	GAC	GAG	CTC	TCT	AGA	GAA	TTC
			*Sal*I		*Sac*I		*Xba*I		*Eco*RI	

GENOTYPE

λ*lac*5 Δ*shn*dIIIλ2-3 *sr*Iλ3° Δ*ssl*Iλ1-2 c*Its*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

GENETIC CHARACTERISTICS

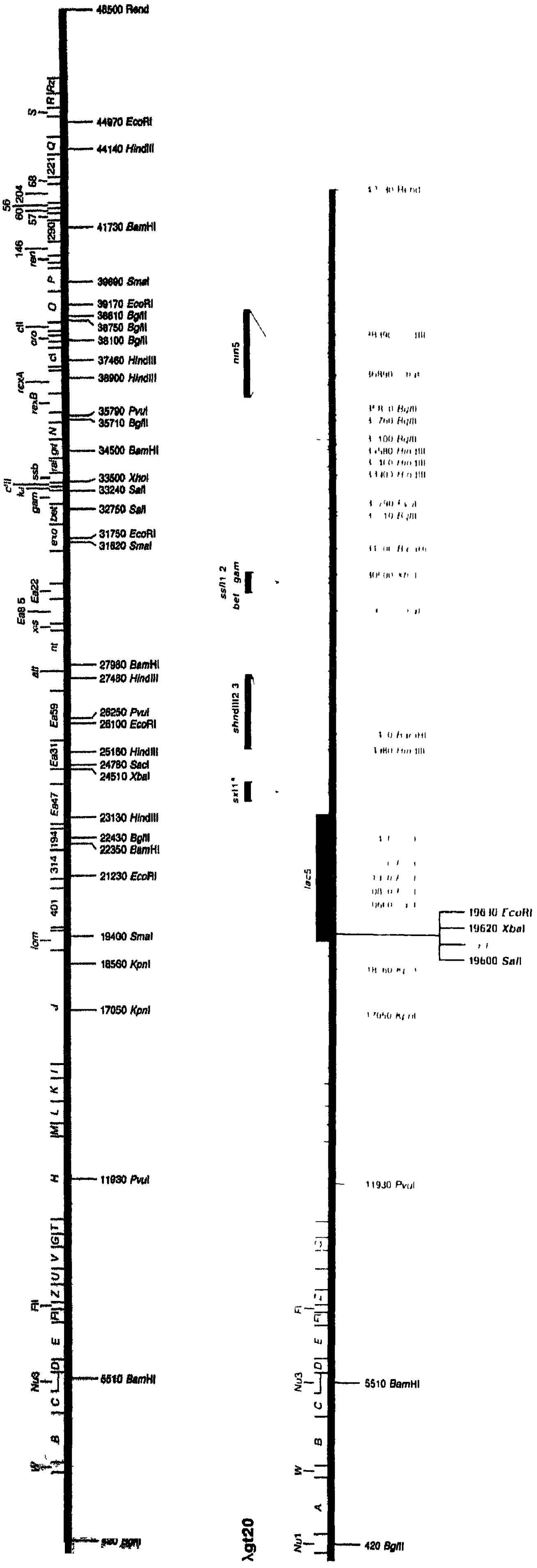
Amber mutations	*S*am100
Suppressor required	*supF*
Deletions	*shn*dIIIλ2-3, *ssl*Iλ1-2, *nin*5
Substitutions	*lac*5
Insertions	no
red/*gam* status of vector	*red*$^-$ *gam*$^-$
red/*gam* status of recombinants	*red*$^-$ *gam*$^-$
chi site present in recombinants	probably
Propagates on *recA*$^-$ hosts	no
Spi selection	no
Recommended hosts	Y1090*hsdR*

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	Size (kb) right arm	Size (kb) insert	Recombinants
*Sal*I *Eco*RI	Insertion	19.5	23.7	0–7.7	Lac$^-$

REFERENCE

Han et al. (1987)


48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglI
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24760 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
S
R
Rz
Q
221
68
204
56
60
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
kil
ral
ssb
cIII
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
lom
J
I
K
L
M
H
G
T
V
U
Z
FII
FI
E
D
Nu3
C
B
W
A
Nu1
nin5
ssI1 2
bet gam
shndIII2 3
sxI1°
λgt20
lac5
19610 EcoRI
19620 XbaI
19600 SalI
11930 PvuI
5510 BamHI
420 BglII

λgt20 and λgt21

λgt20 and λgt21 were derived from λgt18 and λgt19, respectively The polycloning-site sequences are identical to those in λgt18 and λgt19 Two changes were made A synthetic *chi* site was introduced, and a *Sac*I and *Xba*I site were eliminated These manipulations resulted in deletion of about 500 bp in the bacteriophage λ DNA to the right of *lac*5 This allows larger inserts (up to 8 2 kb) than in the λgt18 and λgt19 vectors λgt20 and λgt21 are used for cDNA cloning and immunological screening using any one of three unique restriction sites (*Sal*I, *Xba*I, *Eco*RI) in *lacZ* The only difference between λgt20 and λgt21 is the orientation of the polycloning site The DNA and amino acid sequences introduced are

	Glu	Phe	Ser	Arg	Glu	Leu	Val	Asp	Gln	Phe
λgt20	GAA	TTC	TCT	AGA	GAG	CTC	GTC	GAC	CAA	TTC
	*Eco*RI		*Xba*I		*Sac*I		*Sal*I			

	Glu	Leu	Val	Asp	Glu	Leu	Ser	Arg	Glu	Phe
λgt21	GAA	TTG	GTC	GAC	GAG	CTC	TCT	AGA	GAA	TTC
			*Sal*I		*Sac*I		*Xba*I		*Eco*RI	

GENOTYPE

λ*lac*5 Δ*sx*Iλ1° *chi* Δ*shn*dIIIλ2-3 *sr*Iλ3° Δ*ssl*Iλ1-2 c*I*ts857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

GENETIC CHARACTERISTICS

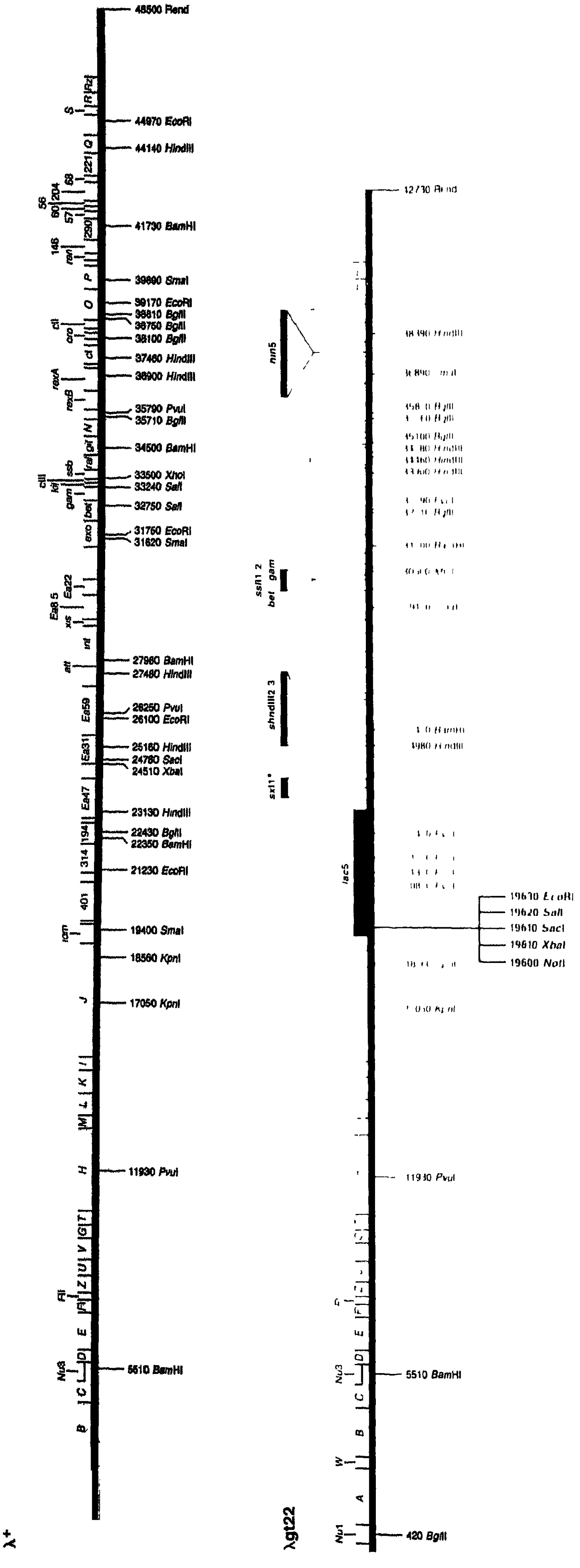
Amber mutations	*S*am100
Suppressor required	*supF*
Deletions	*sx*Iλ1°, *shn*dIIIλ2-3, *ssl*Iλ1-2, *nin*5
Substitutions	*lac*5
Insertions	no
red/*gam* status of vector	*red*⁻ *gam*⁻
red/*gam* status of recombinants	*red*⁻ *gam*⁻
chi site present in recombinants	yes
Propagates on *recA*⁻ hosts	no
Spi selection	no
Recommended hosts	Y1090*hsdR*

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Sal*I *Xba*I *Eco*RI	Insertion	19 5	23 2	0–8 2	Lac⁻

REFERENCE

Han and Rutter (1988)


λ+
λgt22
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
S
R
Rz
Q
221
68
56
60
204
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
pif
ssb
raf
cIII
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
rorn
J
I
K
L
M
H
T
G
V
U
Z
FII
FI
E
D
Nu3
C
B
12730 Rend
nin5
ssII1 2
bet gam
shndIII2 3
sx11*
lac5
19630 EcoRI
19620 SalI
19610 SacI
19610 XbaI
19600 NotI
11930 PvuI
5510 BamHI
420 BglII
W
A
Nu1

λgt22 and λgt23

λgt22 and λgt23 are insertion vectors; cDNA fragments up to 8 2 kb can be cloned into a polycloning site within *lacZ*, allowing expression of fusion proteins detectable by immunological screening The polycloning site inserted at the *Eco*RI site in *lacZ* contains five unique cloning sites *Not*I, *Xba*I, *Sac*I[1], *Sal*I, and *Eco*RI. The two vectors differ only in the orientation of the polycloning site. They have been designed to allow directional cloning of full-length cDNA clones Cloning cDNA in a defined orientation using the *Not*I and *Sal*I sites minimizes the probability of partial cDNA clones, because these sites are seldom found in eukaryotic DNA

GENOTYPE

λ*lac*5 Δ*sx*Iλ1° *chi* Δ*shn*dIIIλ2-3 *sr*Iλ3° Δ*ssl*Iλ1-2 c*Its*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

GENETIC CHARACTERISTICS

Amber mutations	*S*am100
Suppressor required	*supF*
Deletions	*sx*Iλ1°, *shn*dIIIλ2-3, *ssl*Iλ1-2, *nin*5
Substitutions	*lac*5
Insertions	no
red/gam status of vector	*red*⁻ *gam*⁻
red/gam status of recombinants	*red*⁻ *gam*⁻
chi site present in recombinants	yes
Propagates on *recA* hosts	no
Spi selection	no
Recommended hosts	Y1090*hsdR*

CLONING SITES

Site	Insertion/ substitution	Size (kb)			Recombinants
		left arm	right arm	insert	
*Not*I *Xba*I *Sac*I[1] *Sal*I *Eco*RI	Insertion	19 5	23 2	0–8 2	Lac⁻

REFERENCES

Han and Rutter (1987, 1988)

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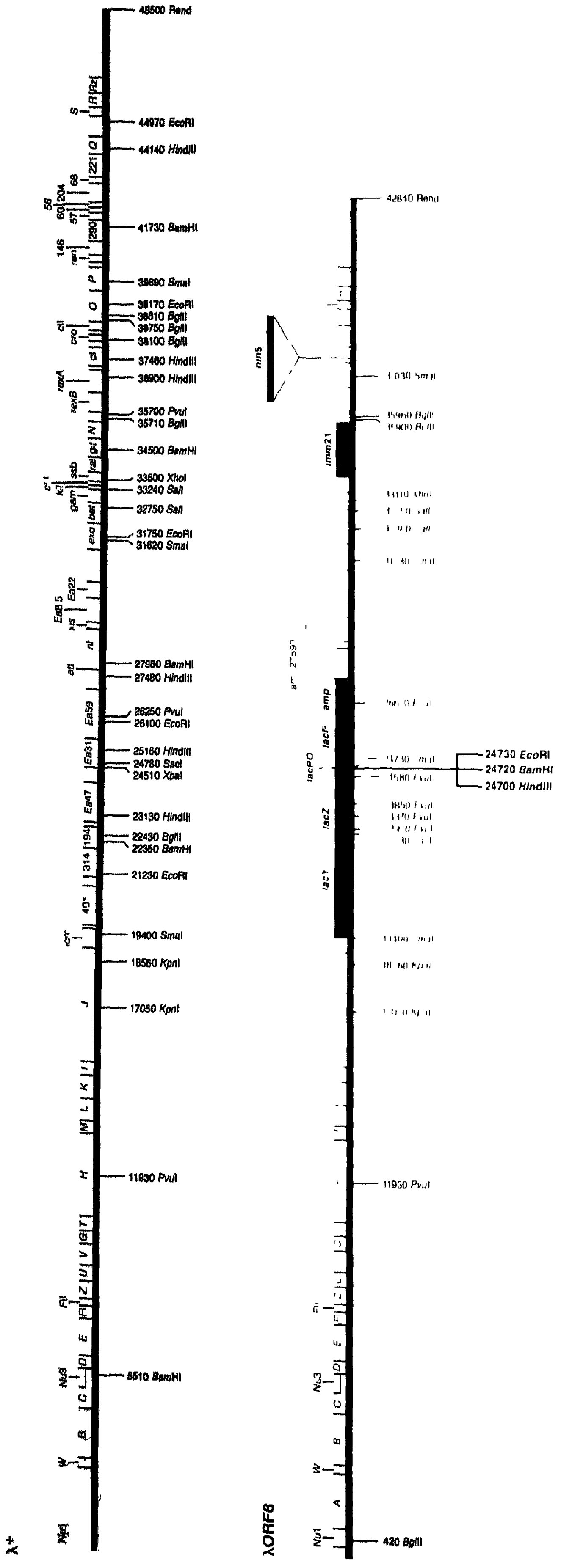
[1] In earlier versions of these vectors, there was an additional *Sac*I site in *lacZ* If you plan to use the *Sac*I site, make sure you have the version from which the extra *Sac*I site has been removed Destruction of the *Sac*I site in *lacZ* resulted in bacteriophages that give rise to very light blue plaques as compared with plaques of bacteriophages that retain the *Sac*I site (J Han, pers. comm)

 λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24780 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
Nu1
W
B
C
Nu3
D
E
FI
FII
Z
U
V
G
T
H
M
L
K
I
J
S
R
Rz
Q
221
68
56
204
60
57
290
146
ren
P
O
cII
cro
cI
rexA
rexB
N
ssb
c
kil
gam
bet
exo
Ea22
Ea8.5
xis
int
att
Ea59
Ea31
Ea47
194
314
401
λORF8
42810 Rend
nin5
11930 PvuI
imm21
amp
lacPO
lacZ
lacY
24730 EcoRI
24720 BamHI
24700 HindIII
420 BglII
Nu1
A
W
B
C
Nu3
D
E
FI

λORF8 can be used for construction of cDNA libraries The bacteriophage contains the *lac* operon DNA and amp^r Unique *Hin*dIII, *Bam*HI, and *Eco*RI sites are located in the 5′ *lacZ* coding region DNA fragments up to 9 kb in length can be directionally cloned into these sites If a coding region is in-frame with *lacZ* sequences, fusion proteins may be expressed Therefore, this vector can be used for antibody screening Recombinants are expected to be Lac$^-$ if the cloned DNA is not in-frame with *lacZ* or if termination codons are present The vector and recombinants with in-frame fusions will be Lac$^+$ The right arm contains an amber codon in *int* at codon 300, has the *nin*5 deletion, and is *imm*21

GENOTYPE

λ*sbh*Iλ1° ⟨*lacY lacZ srI lacZ*° pUC8 polycloning sites $lacI^{q1}$ *amp*⟩ *sbh*Iλ3° *int*am *sr*Iλ3° *imm*21 *sr*Iλ4° *nin*5 *shn*dIIIλ6° *sr*Iλ5°

GENETIC CHARACTERISTICS

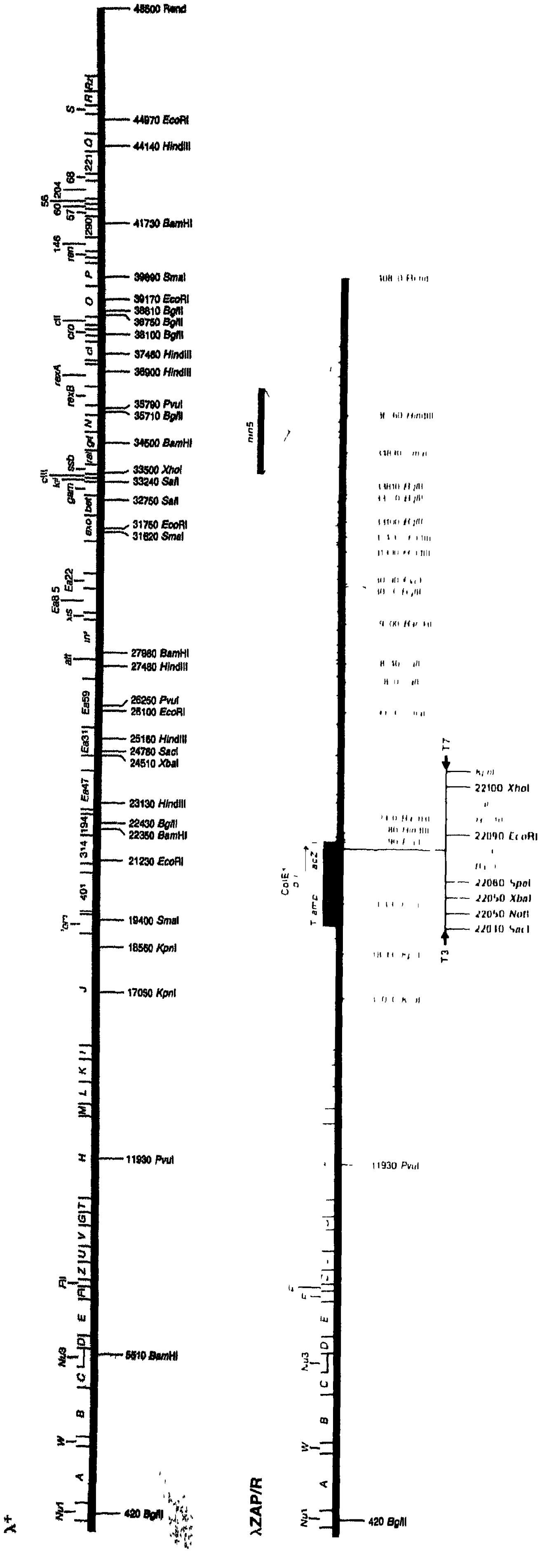
Amber mutations	*int*am
Suppressor required	no
Deletions	*nin*5
Substitutions	($lacY^+$, $lacZ^+$, pUC8 polycloning sites, $lacI^{q1}$, amp^r), *imm*21
Insertions	no
red/*gam* status of vector	red^+ gam^+
red/*gam* status of recombinants	red^+ gam^+
chi site present in recombinants	no
Propagates on *recA*$^-$ hosts	yes
Spi selection	no
Recommended hosts	MC1061 MBM7014 5

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Eco*RI + *Bam*HI *Eco*RI + *Hin*dIII *Bam*HI + *Hin*dIII	Insertion	24 7	18 1	0–9	Lac$^-$ (some Lac$^+$)

REFERENCES

Meissner et al (1987), M Berman (pers comm)


λ+
48500 Rend
44970 EcoRI
44140 HindIII
41730 BamHI
39890 SmaI
39170 EcoRI
38810 BglII
38750 BglII
38100 BglII
37460 HindIII
36900 HindIII
35790 PvuI
35710 BglII
34500 BamHI
33500 XhoI
33240 SalI
32750 SalI
31750 EcoRI
31620 SmaI
27980 BamHI
27480 HindIII
26250 PvuI
26100 EcoRI
25160 HindIII
24760 SacI
24510 XbaI
23130 HindIII
22430 BglII
22350 BamHI
21230 EcoRI
19400 SmaI
18560 KpnI
17050 KpnI
11930 PvuI
5510 BamHI
420 BglII
λZAP/R
nin5
ColE1
lacZ
T7
T3
22100 XhoI
22090 EcoRI
22080 SpeI
22050 XbaI
22050 NotI
22010 SacI
11930 PvuI
420 BglII
Nu3
Nu1

λZAP is an insertion vector equipped with multiple cloning sites within plasmid sequences that can be excised in vivo and converted to a plasmid vector, Bluescript SK (M13−) The plasmid vector is designed to facilitate DNA and RNA sequencing and synthesis of RNA probes utilizing bacteriophage T7 and T3 promoters flanking the cloned fragment In addition, unidirectional deletions, site-directed mutagenesis, expression of fusion proteins, and production of capped RNA transcripts are all readily obtained

DNA fragments up to 10 kb in length can be inserted in unique *Xho*I, *Eco*RI, *Spe*I, *Xba*I, *Not*I, or *Sac*I sites By partially filling recessed termini (see Appendix F), *Sau*3AI, *Mbo*I, *Bgl*II, *Bam*HI, or *Hin*dIII fragments can be cloned The polycloning site is located in the amino-terminal portion of *lacZ'*, often leading to a Lac$^-$ phenotype on isopropylthio-β-D-galactoside/X-gal If the cloned DNA is in-frame with the *lacZ* sequence, a fusion protein may be expressed and detected by antibody screening

The Bluescript SK (M13−) plasmid carrying the cloned DNA is excised in the presence of f1 or M13 helper bacteriophages λZAP contains two signals necessary for excision, initiator (I) and terminator (T), where f1 (+)-strand DNA synthesis initiates and terminates, respectively The orientation of Bluescript in λZAP/R is shown in the map In λZAP/L, the orientation of Bluescript is reversed

λZAPII is equivalent to λZAP except that the *S*am100 mutation has been removed This change allows better growth of the bacteriophage, which in turn causes plaques to become blue much sooner (H Short, pers comm)

λZAP/R

GENOTYPE

λ*sbh*Iλ1° *chi*A131 ⟨T *amp* ColE1 *ori lacZ'* T3 promoter–polycloning site–T7 promoter I⟩ *sr*Iλ3° *c*I*ts*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

GENETIC CHARACTERISTICS

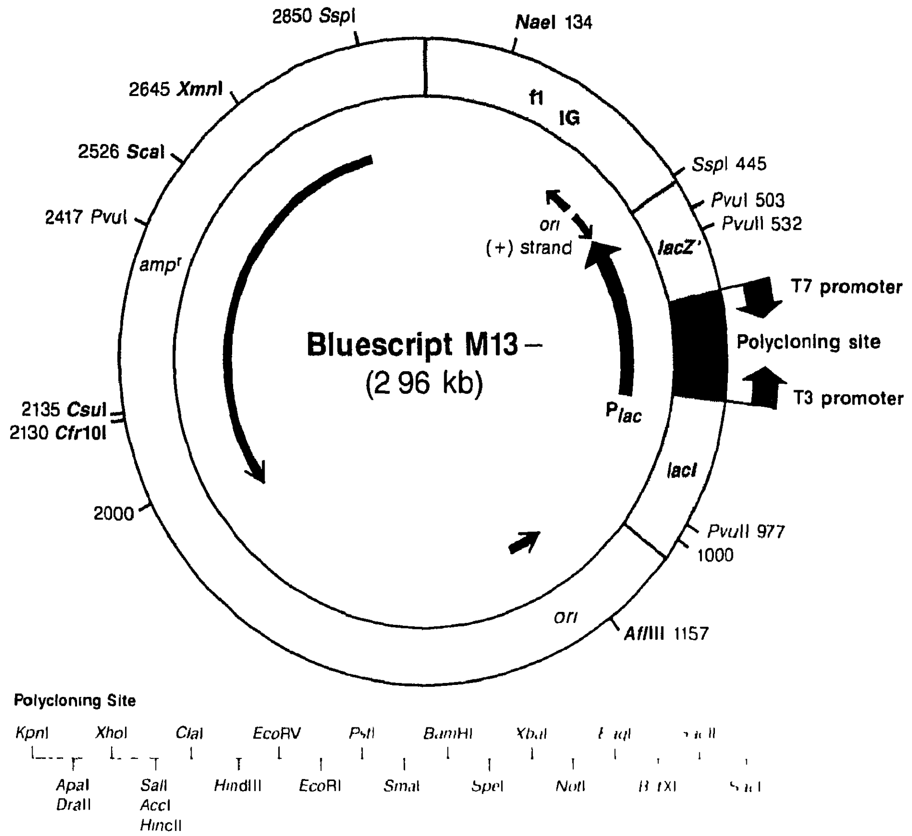
Amber mutations	*S*am100
Suppressor required	*supF*
Deletions	*nin*5
Substitutions	T *amp*^{r} ColE1 *ori lacZ'* (bacteriophage T3 promoter, polycloning site, bacteriophage T7 promoter) I
Insertions	no
red/gam status of vector	*red*$^+$ gam$^+$
red/gam status of recombinants	*red*$^+$ gam$^+$
chi site present in recombinants	yes
Propagates on *recA*$^-$ hosts	yes
Spi selection	no
Recommended hosts	BB4 (λZAP, λZAPII) XL1-Blue (λZAPII)

CLONING SITES

Site	Insertion/ substitution	Size (kb) left arm	right arm	insert	Recombinants
*Sac*I *Not*I *Xba*I *Spe*I *Eco*RI *Xho*I	Insertion	21 8	18 1	0–10	Lac$^-$

REFERENCE

Short et al (1988)



In Bluescript SK (M13) the *SacI* site lies immediately downstream from the bacteriophage T3 promoter and the *KpnI* site lies immediately downstream from the bacteriophage T7 promoter In Bluescript KS (M13) the polycloning site is in the opposite orientation

CHOOSING A BACTERIAL HOST FOR BACTERIOPHAGE λ VECTORS

When using specialized vectors such as bacteriophage λgt11, it is necessary to use particular strains of *E coli* that have been designed to serve as specific hosts. Table 2.2 provides descriptions of some of the genetic markers found in these strains. The strains themselves are listed in Table 2 3 and are more fully described at appropriate places in this manual.

Restriction and Modification

For less specialized vectors, there is a wide choice of strains of *E coli* that support the growth of bacteriophage λ. All of these strains are derivatives of *E coli* K12, which codes for a restriction enzyme (*Eco*K) that cleaves DNA containing unmodified *Eco*K recognition sites Although the DNA of the vector can be protected by growing the bacteriophage in a modifying strain of *E coli,* the foreign DNA used to construct recombinants will still be vulnerable to attack by the *Eco*K restriction enzyme Recombinant bacteriophages should therefore be propagated in a strain of bacteria deficient in restriction (r_k^-) *hsdR*$^-$ strains can modify *Eco*K sites but are defective in restriction (r_k^- m_k^+), *hsdS*$^-$ strains are deficient in both restriction and modification (r_k^- m_k^-)

Amber Suppressors

At one time, it was mandatory for bacteriophage λ vectors to carry amber mutations in genes coding for coat proteins. It was believed that such mutations might reduce the risk of recombinant bacteriophages spreading from the laboratory into field strains of *E coli.* Hosts for these vectors carry one or two strong amber suppressors—*supE* and *supF*—that insert glutamine and tyrosine, respectively, at UAG codons Note that these suppressors are not interchangeable· Some amber mutations in bacteriophage λ vectors are suppressed only by *supE* (e.g , *P*am3) and others only by *supF* (e g , *S*am7 and *S*am100) Since the presence of suppressors does not affect the growth of bacteriophage λ vectors that do not carry amber mutations, we recommend the use of a strain of *E. coli* such as LE392 (*supE supF hsdR*) for routine assay and propagation of bacteriophage λ

Recombination Systems

E coli codes for several pathways that catalyze recombination between homologous sequences of DNA. Because the genomic DNA of many eukaryotes contains sequences that are ideal substrates for homologous recombination, it might be expected that eukaryotic DNAs cloned in bacteriophage λ vectors would undergo rearrangement during propagation in *E. coli* Fortunately, such rearrangements appear to be rare, even in *rec*$^+$ strains of *E. coli.* The few reports of instability (see, e g , Lauer et al. 1980) stand in sharp contrast to the multitude of descriptions of cloned DNA sequences that have not undergone any detectable rearrangement during propagation in *E. coli.* If rearrangement or deletion of cloned sequences is a problem, choose a bacteriophage λ vector that gives rise to recombinants that can be propagated in a *rec*$^-$ host (e g , Charon 32–35 and 40 and λsep6-lac5, which give rise to recombinants that are *red* *gam*$^+$) Recombinants that may be vulnerable to rearrangement should be propagated in a host (e g , CES201) that is defective in the *recA*, *recBC*, and *recF* pathways *rec* cells are usually enfeebled and will be outgrown by hardier *rec*$^+$ revertants and pseudorevertants It is therefore important to use cultures that have been freshly grown from single colonies of bacteria and to check that the bacteria in the culture are sensitive to ultraviolet light (*rec* cells are sensitive to ultraviolet light because they are unable to repair UV-induced damage to their DNA) (see page 2 96)

Note It is not advisable to attempt to propagate *red* *gam* recombinants in *recA* *recBC* strains of *E coli* Not only do these bacterial strains grow comparatively poorly, but they also produce extremely meager yields of bacteriophage λ particles

TABLE 2.2 Genetic Markers in *E. coli* Host Strains

supE *supF*	Bacterial genes encoding suppressor tRNAs for amber mutations *supE* inserts a glutamine residue at the codon UAG, *supF* inserts a tyrosine
hflA	"High frequency lysogenization " Absence of the *hflA* gene product enhances the stability of the *c*II gene product, which in turn results in increased synthesis of the *c*I gene product Bacteriophages capable of lysogenizing do so at very high efficiency (>99%) in strains carrying the *hflA* mutation (Belfort and Wulff 1973, Hoyt et al 1982)
recA	The major bacterial gene involved in recombination by promoting transfer of strands between DNA molecules The *recA* gene product is required for the lytic growth of *red gam* bacteriophage λ (for a review, see Radding 1982)
recB *recC*	Bacterial genes involved in recombination They encode exonuclease V, an enzyme that inhibits late bacteriophage λ replication The bacteriophage λ *gam* gene product inhibits the *recBC* enzyme (Enquist and Skalka 1973)
sbcA	Gene involved in the *recE* recombination pathway The *sbcA* mutation improves the growth of *recB* mutants (Kushner et al 1974)
sbcB	A bacterial gene that encodes exonuclease I, an enzyme that is part of the *recF* pathway The *sbcB* mutation improves the growth of *recB* and *recC* mutants (Kushner et al 1971, Leach and Stahl 1983)
recD	The *recD* gene product is a component of the *recBC* enzyme complex and is necessary for the expression of the enzyme's nucleolytic activities (Amundsen et al 1986) Spi$^-$ derivatives of bacteriophage λ show improved growth on *recD*$^-$ hosts Furthermore, the presence of the mutation diminishes the loss from genomic DNA libraries of recombinant bacteriophages that carry DNA segments containing inverted repetitions (Wyman et al 1985, 1986, Patterson and Dean 1987)
lacI	The *E coli lacI* gene encodes the *lac* repressor The *lacI*^{q} mutation increases the amount of *lac* repressor made in the cell (Muller-Hill et al 1968)
lacZ	*E coli* gene encoding β-galactosidase *lacZ*ΔM15 encodes an enzymatically inactive β-galactosidase lacking amino acids 11–41 The amino-terminal fragments of β-galactosidase produced by various vectors can associate with the *lacZ*ΔM15 products to form enzymatically active proteins (Beckwith 1964, Ullmann and Perrin 1970)
hsdR	Bacterial gene encoding one subunit of the enzyme *Eco*K, a type I restriction enzyme This subunit is responsible for cutting DNA *hsdR* strains methylate but do not restrict DNA (for a review, see Yuan 1981)
hsdS	Gene encoding the subunit of the restriction enzyme that recognizes the host DNA sequence The *hsdS* gene product also functions as the site recognition part of the methylase *hsdS* strains are defective in both restriction and methylation (for a review, see Yuan 1981)
hsdM	Bacterial gene encoding the subunit of *E coli* methylase that methylates *Eco*K restriction sites The *Eco*K restriction enzyme does not recognize sites containing methylated adenines The *hsdS* gene encodes the other methylase subunit (for a review, see Yuan 1981)
mcrB	Bacterial gene encoding a restriction enzyme that recognizes *Alu*I-methylated DNA sites If *Hin*dIII sites in a vector are protected by methylation in vitro with M *Alu*I methylase, *mcrB* strains should be used (Raleigh and Wilson 1986)
lon	A bacterial gene encoding an ATP-dependent protease
rpsL	Gene encoding a ribosomal protein Streptomycin-resistant mutants have a mutation in *rpsL*
dam	A bacterial gene encoding the *dam* methylase, an enzyme that methylates at the N^6 position of adenine in the sequence 5′ GATC 3′ (Hattman et al 1978) *Bcl*I will not cut DNA unless the DNA is isolated from a *dam*$^-$ strain
tonA	*tonA* mutants are resistant to bacteriophage T1 infection
Tn*10*	A transposon encoding resistance to tetracycline When Tn*10* resides in an F′ episome, tetracycline can be used to select cells retaining the F′
pMC9	A plasmid encoding *lacI*^{q}

TABLE 2.3 ***E. coli* Strains Used to Propagate Bacteriophage λ Vectors**

Phenotype	Relevant genotype	Strain	Uses	References
	sup$^\circ$	R594	Nonpermissive for vectors carrying amber mutations	Campbell (1965), Borck et al (1976)
r_k^-	*supE hsdR*	MM294, Q358, C600 (BNN93), K802	Permissive for vectors carrying amber mutations Will modify but not restrict DNA	Appleyard (1954), Wood (1966), Meselson and Yuan (1968), Karn et al (1980), Young and Davis (1983a)
r_k^-	*supE supF hsdR*	LE392, ED8654	Permissive for vectors carrying amber mutations Will modify but not restrict DNA	Borck et al (1976), Murray et al (1977)
r_k^-	*supF hsdR*	NM538	Permissive for vectors carrying amber mutations Will modify but not restrict DNA	Frischauf et al (1983)
r_k^- Rec$^-$	*supE supF hsdR recA*	NM531	Permissive for vectors carrying amber mutations Will modify but not restrict DNA *recA*$^-$ strain	Arber et al (1983)
r_k^- Rec$^-$ Sbc$^-$	*hsdR recA recBC sbcB*	CES201	Will modify but not restrict DNA *recA*$^-$ strain For growth of Spi$^-$ bacteriophage	Wyman and Wertman (1987)
r_k^- RecF$^+$	*hsdR recBC sbcB*	CES200	Will modify but not restrict DNA For growth of Spi$^-$ bacteriophage	Nader et al (1985)
r_k^- RecE$^+$	*hsdR recBC sbcA*	NM519	Will modify but not restrict DNA For growth of Spi$^-$ bacteriophage	Arber et al (1983)
r_k^- RecD$^-$	*supE supF hsdR recD*	TAP90	Used for production of high-titer lysates For growth of Spi$^-$ bacteriophage Will modify but not restrict DNA	Patterson and Dean (1987)
r_k^- McrB$^-$	*hsdR mcrB Δlac*	MBM7014 5 MC1061	Used for bacteriophage λORF8 libraries when *Alu*I methylation is used	Raleigh and Wilson (1986), Meissner et al (1987)
r_k^- Lac$^-$ Lon$^-$	*hsdR supF Δlac Δlon* pMC9	Y1090*hsdR*	Permissive for growth of λgt11 and λgt18–23 Contains high levels of *lac* repressor Is deficient in the *lon* protease Will modify but not restrict DNA	Young and Davis (1983b), Jendrisak et al (1987)
r_k^- Hfl$^-$	*supE hsdR hflA*	BNN102 (C600*hflA*)	Will modify but not restrict DNA Nonpermissive for λgt10 Permissive for recombinants that have foreign DNA inserted in the *c*I gene	Young and Davis (1983a)

r_k^- *lacZ*ΔM15	*supE supF hsdR* F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	BB4	Permissive for vectors carrying amber mutations Will modify but not restrict DNA Permits α-complementation of β-galactosidase	Bullock et al (1987)
r_k^- *lacZ*ΔM15 Rec$^-$	*supE hsdR lac*$^-$ F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	XL1-Blue	Will modify but not restrict DNA Permits α-complementation of β-galactosidase *recA*$^-$ strain	Bullock et al (1987)
Lac$^-$	*supE* Δ(*lac-pro*) F′ *proAB*$^+$ *lacZ*$^-$	CSH18	Permissive for vectors carrying amber mutations Used to screen recombinants made in vectors carrying a *lacZ* gene	Miller (1972), Williams and Blattner (1979)
r_B^- m_B^- Rec$^-$	*hsdS recA*	HB101	Nonpermissive for vectors carrying amber mutations Will not modify or restrict DNA *recA*$^-$ strain	Boyer and Roulland-Dussoix (1969), Bolivar and Backman (1979)
r_B^- m_B^-	*supE supF hsdS*	DP50*supF*	Permissive for vectors carrying amber mutations Will not modify or restrict DNA	Leder et al (1977), B Bachmann (pers comm)
r_B^- m_B^- Rec$^-$	*supE supF hsdS recA*	ED8767	Permissive for vectors carrying amber mutations Will not modify or restrict DNA *recA*$^-$ strain	Murray et al (1977)
C strain		C-1a	Nonpermissive for vectors carrying amber mutations Used for complementation test with amber mutants of bacteriophage λ	Bertani and Weigle (1953), Borck et al (1976)
r_k^- (P2)	*supE hsdR* (P2)	Q359	Used for selection of Spi$^-$ bacteriophage Will modify but not restrict DNA Permissive for vectors carrying amber mutations	Karn et al (1980)
r_k^- (P2)	*supF hsdR* (P2cox)	NM539	Used for selection of Spi$^-$ bacteriophage Will modify but not restrict DNA Permissive for vectors carrying amber mutations	Frischauf et al (1983)
λ*E*am	(N205 *recA* [λ*imm*434 c*I*ts *b*2 *red* *E*am *S*am/λ])	BHB2688	Used to prepare packaging extracts	Hohn and Murray (1977), Hohn (1979)
λ*D*am	(N205 *recA* [λ*imm*434 c*I*ts *b*2 *red* *D*am *S*am/λ])	BHB2690	Used to prepare packaging extracts	Hohn and Murray (1977), Hohn (1979)
λ*cos*2	*E coli* C (λ*cos*2 Δ*B* *xis red gam*am210 c*I*ts857 *nin* *S*am/λ)	SMR10	Used to prepare packaging extracts	Rosenberg (1985)

Bacteriophage λ Growth, Purification, and DNA Extraction

PLAQUE PURIFICATION OF BACTERIOPHAGE λ

Preparation of Plating Bacteria

1. Place 50 ml of sterile rich medium (e.g., NZCYM or LB) supplemented with 0.2% maltose in a sterile 250-ml flask and inoculate with a single bacterial colony. Grow the culture overnight at 37°C with moderate agitation (250 cycles/minute in a rotary shaker). Bacteria grown in the presence of maltose adsorb bacteriophage λ more efficiently than if maltose is not present, the sugar induces the maltose operon, which contains the gene (*lamB*) that codes for the bacteriophage λ receptor.

2. Centrifuge the cells at 4000*g* for 10 minutes at room temperature.

3. Discard the supernatant, and resuspend the cell pellet by vortexing in sterile 0.01 M $MgSO_4$ (~20 ml). More consistent results are obtained if the resuspended cells are diluted to an appropriate density (usually $OD_{600} = 2$, i.e., ~1.6×10^9 cells/ml).

   Because the relationship between the OD_{600} and the number of viable cells per milliliter varies substantially from strain to strain, it is essential to calibrate the spectrophotometer by measuring the OD_{600} of a growing culture of the particular strain of *E. coli* at different times in its growth cycle and determining the number of viable cells at each of these times by plating dilutions of the culture on LB agar plates in the absence of antibiotics.

4. *Optional.* Transfer the cell suspension to a 100-ml sterile flask and incubate for 1 hour at 37°C with moderate agitation (250 cycles/minute in a rotary shaker).

   Some investigators, but not all, find that this step improves the viability of the stored cell suspension.

5. Store the cell suspension at 4°C. The suspension may be used for up to 3 weeks. However, the highest plating efficiencies are obtained when freshly prepared cells are used.

   Severely enfeebled strains of *E. coli* (e.g., *recA*$^-$ strains) lose viability quickly when stored under conditions of starvation at 4°C. Fresh cultures of such strains (whose genotypes should be revalidated frequently) should be used for plating.

Plating Bacteriophage λ

A plaque derives from infection of a single bacterium by a single virus particle. The progeny particles infect neighboring bacteria, which in turn release another generation of daughter virus particles. If bacteria are growing in semisolid medium (containing, e.g., agarose or agar), the diffusion of the progeny virus particles is limited. Under these circumstances, the result of successive rounds of infection is a spreading zone of lysis that eventually becomes visible to the naked eye as a clear area in an otherwise turbid background of bacterial growth. Because each plaque contains the progeny of a single bacteriophage particle, it is possible to obtain populations of bacteriophages whose members are essentially genetically identical to one another.

1. Prepare tenfold serial dilutions of bacteriophage stocks in SM.

2. Dispense 0.1 ml of each dilution to be assayed into a sterile tube (13 mm × 100 mm)

3. Add 0.1 ml of plating bacteria to each tube. Mix by shaking or vortexing. Incubate for 20 minutes at 37°C to allow the bacteriophage particles to adsorb to the bacteria

4. Add 3 ml of molten (47°C) agar or agarose (0.7%) to the first tube, vortex it gently, and immediately pour the entire contents of the tube onto the center of a labeled plate containing 30–35 ml of hardened bottom agar medium (NZCYM or LB). Try to avoid creating air bubbles Swirl the plate gently to ensure an even distribution of bacteria and top agar/agarose Repeat with the second and subsequent tubes on fresh plates

   The plates containing hardened bottom agar medium should be removed from storage at 4°C and equilibrated at room temperature for 1–2 hours before use This eliminates problems of condensation and allows the top agar to spread across the entire surface of the plate before it sets

5. Close the plates and let them stand for 5 minutes at room temperature to allow the top agar/agarose to harden Invert the plates and incubate at 37°C Plaques begin to appear after about 7 hours of incubation and should be counted or picked after 12–16 hours.

Notes

i Although there can be considerable variability, stocks of bacteriophage λ prepared from liquid cultures of infected bacteria or from confluently lysed plates contain between 10^9 and 10^{11} pfu/ml.

ii Freshly poured plates should not be used for plaque assay of bacteriophage λ. During incubation at 37°C, droplets of moisture that form on the surface of the top agar cause the developing plaques to streak and run into one another This problem can be avoided by storing the plates for 2–4 days at room temperature before use or by drying the plates with the lids ajar for 1 hour in a 37°C incubator. In an emergency, when there is no time to dry the plates, remove any droplets of water from the lids of the plates after the top agar has set and insert a piece of circular filter paper into each lid. During incubation of the inverted plates at 37°C, the filter paper absorbs much of the humidity and keeps streaking to a minimum.

iii Plaques produced by different strains of bacteriophage λ or by different recombinants constructed within a single strain vary in size and appearance. For example, recombinants of Charon 28 and 30 containing foreign DNA sequences that carry a *chi* site produce much larger plaques than those that do not. However, using the protocol given above, in which the bacteriophage particles are allowed to adsorb to the bacterial suspension before plating, there should be little variation between plaques formed by members of genetically homogeneous stocks of the bacteriophage The appearance of plaques is also influenced by the rate of growth of the bacterial lawn, the concentration of agar/agarose, the freshness of the plates, and the humidity of the incubator Small plaques are favored by vigorous bacterial growth, high concentrations of top agar/agarose, dry plates, and a dry atmosphere in the incubator With experience, these conditions can be adjusted to suit the task at hand For example, when screening libraries by hybridization, it is often desirable to restrict the size of the plaques so that the maximum number of bacteriophages can be screened per plate In this case, use well-aged plates and use slightly more bacteria than usual for each lawn. Under other conditions—for example, when using enfeebled *red*$^-$ *gam*$^-$ strains of bacteriophage λ—it may be necessary to reduce the bacterial inoculum and/or to use a lower (0 6%) concentration of top agar/agarose to obtain plaques of an acceptable size. Bacteriophage λ plaques do not increase in size after the bacterial lawn is fully grown and the cells have reached stationary phase

Picking Bacteriophage λ Plaques

1. Place 1 ml of SM in a polypropylene tube (13 mm × 100 mm) Add 1 drop of chloroform

2. Using a pasteur pipette equipped with a rubber bulb, or a micropipette, stab through the chosen plaque into the hard agar beneath Apply mild suction so that the plaque, together with the underlying agar, is drawn into the pipette.

   Because bacteriophage λ can diffuse considerable distances through the layer of top agar, choose well-separated plaques For the same reason, it is advisable to pick plaques shortly after the bacterial lawn has grown up and the bacteriophage plaques have first appeared Picking plaques early in their development also reduces the unwelcome possibility of obtaining a bacteriophage stock that contains significant numbers of undesirable mutants

3. Place the fragments of agar in the SM/chloroform prepared in step 1. Let stand at room temperature for 1–2 hours to allow the bacteriophage particles to diffuse out of the agar. An average plaque yields 10^6–10^7 infectious bacteriophage particles, which can be stored indefinitely at 4°C in SM/chloroform without loss of viability

Notes

i It is also possible to isolate plaques by touching a sterile 8-cm wooden stick (also called applicator or orange stick) or a sterile toothpick to the surface of the top agar/agarose in the center of the chosen plaque. Immediately place the stick in SM/chloroform and vortex to dislodge fragments of agar/agarose containing the bacteriophage.

ii Aged batches of chloroform may inactivate bacteriophage λ. This problem can be avoided by using freshly distilled chloroform or chloroform that has been extracted with anhydrous sodium bicarbonate to remove products of photolysis.

PREPARING STOCKS OF BACTERIOPHAGE λ FROM SINGLE PLAQUES

Two methods are commonly used to prepare stocks from single plaques of bacteriophage λ:

- Plate lysates, in which the bacteriophages are propagated in bacteria grown in soft agar
- Small-scale liquid cultures, in which the bacteriophages are propagated in bacteria in liquid medium

Although both methods yield useful bacteriophage stocks, the first has the advantage of allowing the experimenter to determine whether or not the bacteriophages have grown successfully merely by looking at the degree of confluence of the plaques

Plate Lysate Stocks

To achieve maximum yield, the number of bacteriophages plated should be adjusted so that the outer edges of the expanding plaques just touch after approximately 12 hours of incubation An inoculum of 10^5 pfu is usually sufficient to produce confluent lysis of a bacterial lawn growing on a 90-mm plate. By the end of the period of growth, there should be no patches of uninfected bacteria

Two protocols for the preparation of plate lysate stocks are given below Both protocols work equally well Protocol II is more rapid, but it requires additional manipulations In Protocol I, the bacteriophages are eluted from the plate by diffusion into SM, whereas in Protocol II, the bacteriophages are plated in low-percentage top agar/agarose and eluted by scraping the top agar/agarose from the plate and disrupting in SM

PREPARATION OF PLATE LYSATE STOCKS: PROTOCOL I

1. Mix 10^5 pfu of bacteriophage (usually about 1/20 of a resuspended plaque) with 0.1 ml of plating bacteria. Incubate for 20 minutes at 37°C.

   When preparing stocks of strains of bacteriophage λ that grow poorly, the inoculum should be increased to 10^6 pfu.

2. Add 3 ml of molten (47°C) top agar/agarose (0.7%), mix, and pour immediately onto a labeled 90-mm plate containing 30–35 ml of hardened bottom agar. A freshly poured plate equilibrated to room temperature gives the best results, but an older plate (1–4 days old) gives a satisfactory yield.

   Adequate titers of most strains of bacteriophage λ can be obtained by using LB or NZCYM bottom agar. However, the highest yields (Arber et al. 1983) are obtained from LB bottom agar containing
   0.3% glucose
   0.075 mM $CaCl_2$
   0.004 mM $FeCl_3$
   2 mM $MgSO_4$
   Glucose represses the *lamB* gene and reduces the amount of bacteriophage that is lost by adsorption to bacterial debris. Calcium ions also reduce loss by readsorption, whereas ferric ions may stimulate respiration and suppress lysogeny.

   Set up a plate that contains bacteria but no bacteriophages. During incubation, this plate should develop a smooth lawn of confluent bacterial growth, which can serve as a standard if doubt should arise as to whether the absence of bacterial growth on the infected plates is due to lysis or inadequate growth of the host.

3. Incubate the plate for 6–8 hours at 37°C. At the time of harvesting, the plaques should touch one another, and the only visible bacterial growth should be a gauzy webbing that marks the junction between adjacent plaques.

4. Remove the plate from the incubator, add 5 ml of SM, and store at 4°C for several hours with intermittent, gentle shaking.

5. With a pasteur pipette, harvest as much of the SM as possible and place in a polypropylene tube (13 mm × 100 mm).

6. Add 1 ml of fresh SM to the plate and store it for 15 minutes in a tilted position to allow all of the fluid to drain into one area. Again remove the SM and combine it with the first harvest. Discard the plate.

7. Add 0.1 ml of chloroform to the pooled SM, vortex briefly, and centrifuge at 4000*g* for 10 minutes at 4°C.

8. Recover the supernatant, add 1 drop of chloroform, and store at 4°C. The titer of the stock should be approximately 10^{10} to 10^{11} pfu/ml, and this usually remains unchanged as long as the stock is stored at 4°C.

PREPARATION OF PLATE LYSATE STOCKS: PROTOCOL II

1. Mix 10^5 pfu of bacteriophage (usually about 1/20 of a resuspended plaque) with 0.1 ml of plating bacteria. Incubate for 20 minutes at 37°C.

   When preparing stocks of strains of bacteriophage λ that grow poorly, the inoculum should be increased to 10^6 pfu

2. Add 3 ml of molten (47°C) top agar/agarose (0.5%), mix, and pour immediately onto a labeled 90-mm plate containing 30–35 ml of hardened bottom agar. A freshly poured plate equilibrated to room temperature gives the best results, but an older plate (1–4 days old) gives a satisfactory yield.

   Low-percentage top agar/agarose (0 5%) is used here so that it can be easily scraped off the plate after the bacteriophages have grown

   Adequate titers of most strains of bacteriophage λ can be obtained by using LB or NZCYM bottom agar However, the highest yields (Arber et al 1983) are obtained from LB bottom agar containing
   0 3% glucose
   0 075 mM $CaCl_2$
   0 004 mM $FeCl_3$
   2 mM $MgSO_4$
   Glucose represses the *lamB* gene and reduces the amount of bacteriophage that is lost by adsorption to bacterial debris Calcium ions also reduce loss by readsorption, whereas the ferric ions may stimulate respiration and suppress lysogeny

   Set up a plate that contains bacteria but no bacteriophages During incubation, this plate should develop a smooth lawn of confluent bacterial growth, which can serve as a standard if doubt should arise as to whether the absence of bacterial growth on the infected plates is due to lysis or inadequate growth of the host

3. Incubate the plate without inversion for 6–8 hours at 37°C Plates are not inverted during incubation to prevent the top agar/agarose from slipping At the time of harvesting, the plaques should touch one another, and the only visible bacterial growth should be a gauzy webbing that marks the junction between adjacent plaques.

4. When confluent lysis has occurred, gently scrape the soft top agar/agarose into a sterile centrifuge tube by using a sterile bent glass rod

5. Add 5 ml of SM to the plate to rinse off any remaining top agar/agarose, and add it to the centrifuge tube.

6. Add 0.1 ml of chloroform to the agar/agarose suspension Mix by rotation or shaking for 15 minutes at 37°C.

7. Centrifuge at 4000*g* for 10 minutes at 4°C.

8. Recover the supernatant, add 1 drop of chloroform, and store at 4°C. The titer of the stock should be approximately 10^{10} to 10^{11} pfu/ml, and this usually remains unchanged as long as the stock is stored at 4°C.

Small-scale Liquid Cultures

This method was originally described by Leder et al. (1977). In general, the yields of bacteriophage λ are lower and more variable than those obtained from plate lysates.

1. Mix 0.1 ml of a fresh, overnight bacterial culture with approximately 10^6 pfu of bacteriophage (usually about 1/5–1/2 of a resuspended plaque) in a sterile culture tube (17 mm × 100 mm) with a loose-fitting cap.

2. Incubate for 15 minutes at 37°C to allow the bacteriophage particles to adsorb

3. Add 4 ml of NZCYM or LB medium and incubate with vigorous shaking until lysis occurs (6–12 hours at 37°C) Shaking can be best accomplished by positioning the tube in a shaker incubator or in a rotating wheel

4. After lysis has occurred, add 2 drops of chloroform and continue incubation for 15 minutes at 37°C

5. Centrifuge at 4000*g* for 10 minutes at 4°C.

6. Recover the supernatant, add 1 drop of chloroform, and store at 4°C. The titer of the stock should be approximately 10^{10} pfu/ml, and this usually remains unchanged as long as the stock is stored at 4°C.

Notes

i. If lysis does not occur or is incomplete, add an equal volume of prewarmed NZCYM or LB medium to the cultures and continue incubation for a further 2–3 hours at 37°C with vigorous agitation (300 cycles/minute in a rotary shaker)

ii Although most strains of bacteriophage λ grow well in liquid culture and routinely yield lysates containing approximately 5 × 10^9 pfu/ml, there are some strains that replicate poorly The problem can often be solved by increasing the ratio of bacteriophage to bacteria in the infection or by growing the infected culture at 39°C—the optimum temperature for growth of the bacteriophage In addition, some workers prefer to carry out the adsorption step at 0°C in the presence of 5 mM $CaCl_2$ (Patterson and Dean 1987) This allows a more uniform infection and synchronous growth of the bacteriophage

Long-term Storage of Bacteriophage λ Stocks

Stocks of most strains of bacteriophage λ are stable for several years when stored at 4°C in SM containing a small amount of chloroform (0.3%) However, many workers have found that some bacteriophage λ recombinants lose viability when stored in this way for a few months This problem can usually be avoided by using freshly distilled chloroform or chloroform that has been extracted with anhydrous sodium bicarbonate to remove products of photolysis. However, we also recommend that the titer of master stocks be checked every 2–3 months and that master stocks of all important bacteriophage λ strains whose genotypes have been verified be stored at −70°C Before storage, add dimethyl sulfoxide (DMSO) to the bacteriophage stock to a final concentration of 7% v/v. Mix gently. Plunge the container into liquid nitrogen. When the liquid has frozen, transfer the container to a freezer at −70°C for long-term storage.

To recover the bacteriophages, scrape the frozen surface of the liquid with a sterile 18-gauge needle. Streak the needle over the surface of a plate containing the appropriate indicator bacteria Incubate for 12 hours at the appropriate temperature to obtain bacteriophage λ plaques. Pick a well-isolated plaque and generate a high-titer stock by one of the methods described earlier.

LARGE-SCALE PREPARATION OF BACTERIOPHAGE λ

Two methods are presented below for the preparation of large quantities of bacteriophage λ.

- Infection at low multiplicity
- Infection at high multiplicity

After infection of bacteria at low multiplicity, a large volume of medium is inoculated with the infected culture. Initially, the concentration of bacteriophages is low, and uninfected cells in the culture continue to divide for several hours However, successive rounds of infection lead to the production of increasing quantities of bacteriophage Eventually, all of the bacteria become infected and complete lysis of the culture occurs Care is required with this method because small changes in the ratio of cells to bacteriophage particles in the initial infection greatly affect the final yield of bacteriophages Furthermore, the optimum ratio varies for different strains of bacteriophage λ and bacteria However, with a little effort, the method can be adapted for use with most combinations of virus and host cells.

The second method, which suffers less from the strain-to-strain variation that can occur during protracted periods of bacteriophage growth, involves infection at high multiplicity and growth of bacteriophages for a short period of time (usually 3–5 hours), by which time the culture is completely lysed.

Infection at Low Multiplicity

There are many variants of this method. In our hands, however, the following protocol for the preparation of bacteriophage λ from a 2-liter culture of infected bacteria gives the best results (Blattner et al. 1977; Maniatis et al. 1978).

1. Place 100 ml of NZCYM in a 500-ml flask and inoculate with a single colony of an appropriate bacterial host Incubate overnight at 37°C with vigorous agitation (300 cycles/minute in a rotary shaker)

2. Read the OD_{600} of the culture. Calculate the cell concentration assuming that 1 $OD_{600} = 8 \times 10^8$ cells/ml.

3. Withdraw four aliquots, each containing 10^{10} cells. Centrifuge at 4000*g* for 10 minutes at room temperature Discard the supernatants.

4. Resuspend each of the bacterial pellets in 3 ml of SM.

5. Add the bacteriophage and mix rapidly. The number of bacteriophage particles used is critical. For strains of bacteriophage λ that grow well (e.g., EMBL3 and 4 and λgt10), 5×10^7 pfu should be added to each suspension of 10^{10} cells; for bacteriophages that grow relatively poorly (e.g., the Charon series), it is better to increase the starting inoculum to 5×10^8 pfu. However, there are no hard and fast rules, and you will probably need to experiment to find the multiplicity that gives the best results under your conditions.

6. Incubate for 20 minutes at 37°C with intermittent shaking.

7. Add each infected aliquot to 500 ml of NZCYM, prewarmed to 37°C in 2-liter flasks. Incubate at 37°C with vigorous shaking Begin to monitor the cultures after 8 hours. Concomitant growth of bacteria and bacteriophages should occur, resulting in lysis of the culture after 9–12 hours A fully lysed culture contains a considerable amount of bacterial debris, which can vary in appearance from a fine, splintery precipitate to much larger, stringy clumps. If the cultures are held up to the light, the schlieren patterns and silky appearance of a dense, unlysed bacterial culture should not be visible.

8. If lysis is not apparent, check a small sample of the cultures for evidence of bacteriophage growth Withdraw into glass tubes two aliquots (1 ml each) of the infected culture. Add 1 or 2 drops of chloroform to one of the tubes Incubate both tubes for 5–10 minutes at 37°C with intermittent shaking Compare the appearances of the two cultures by holding the tubes up to a light. If infection is near completion but the cells have not yet lysed, the chloroform will cause the cells to burst and the turbid culture will clear to the point where it is translucent. In this case, proceed to step 9. If lysis does not occur or is incomplete, the preparation can sometimes be rescued by adding to each of the cultures an additional

500 ml of NZCYM preheated to 37°C. Continue to incubate for a further 2–3 hours at 37°C with shaking as vigorous as possible (300 cycles/minute in a rotary shaker).

9. Add 10 ml of chloroform to each flask, and continue to incubate for a further 10 minutes at 37°C with shaking.

10. Proceed to "Standard Method for Purification of Bacteriophage λ," step 1 (page 2.73)

Infection at High Multiplicity

In this method, bacteriophage λ is prepared from a 2-liter culture of infected bacteria as follows.

1. Place 10 ml of NZCYM in a 50-ml flask and inoculate with a single colony of an appropriate bacterial host. Incubate overnight at the appropriate temperature with vigorous agitation (300 cycles/minute in a rotary shaker).

2. Inoculate 500 ml of NZCYM, prewarmed to 37°C in each of four 2-liter flasks, with 1 ml of the overnight culture per flask Incubate at 37°C with vigorous shaking until the OD_{600} of the cultures reaches 0 5 (3–4 hours).

3. Inoculate each flask with 10^{10} pfu of bacteriophage λ, and continue to incubate at 37°C with vigorous shaking until lysis occurs (usually 3–5 hours). A fully lysed culture contains a considerable amount of bacterial debris, which can vary in appearance from a fine, splintery precipitate to much larger, stringy clumps. If the culture is held up to the light, the schlieren patterns and silky appearance of a dense, unlysed bacterial culture should not be visible.

   To maximize the yield of particular strains of bacteriophage λ, it may be necessary to adjust the multiplicity of infection or the length of incubation

4. Add 10 ml of chloroform to each flask, and continue to incubate for a further 10 minutes at 37°C with shaking

5. Proceed to "Standard Method for Purification of Bacteriophage λ," step 1 (page 2.73).

PURIFICATION OF BACTERIOPHAGE λ

The method given below for purification of bacteriophage particles is essentially that of Yamamoto et al (1970). Over the years, many variants and shortcuts have emerged Three slightly modified procedures are therefore presented following this protocol.

Standard Method for Purification of Bacteriophage λ

1. Cool lysed cultures to room temperature and add pancreatic DNAase I and RNAase, each to a final concentration of 1 μg/ml. Incubate for 30 minutes at room temperature

   Crude commercial preparations of both enzymes are adequate to digest the nucleic acids liberated from the lysed bacteria Without digestion, a significant number of bacteriophage particles become entrapped in the viscous solution of nucleic acids.

2. Add solid NaCl to a final concentration of 1 M (29.2 g per 500 ml of culture) Dissolve by swirling Let stand for 1 hour on ice The addition of NaCl promotes dissociation of bacteriophage particles from bacterial debris and is required for efficient precipitation of bacteriophage particles from polyethylene glycol.

3. Remove debris by centrifugation at 11,000*g* for 10 minutes at 4°C Pool the supernatants in a clean flask.

4. Add solid polyethylene glycol (PEG 8000) to a final concentration of 10% w/v (i e , 50 g per 500 ml of supernatant). Dissolve by slow stirring on a magnetic stirrer at room temperature.

   Some workers prefer to add the polyethylene glycol at the same time as the NaCl (step 2) The centrifugation step (3) may then be omitted Simultaneous addition of polyethylene glycol and NaCl works efficiently only if the bacteriophage grows well and the titer of bacteriophage in the original lysed culture is greater than 2×10^{10} pfu/ml

5. Cool in ice water and let stand for at least 1 hour on ice to allow the bacteriophage particles to form a precipitate.

6. Recover the precipitated bacteriophage particles by centrifugation at 11,000*g* for 10 minutes at 4°C. Discard the supernatant, and stand the centrifuge bottle in a tilted position for 5 minutes to allow the remaining fluid to drain away from the pellet. Remove the fluid with a pipette.

7. Using a wide-bore pipette equipped with a rubber bulb, gently resuspend the bacteriophage pellet in SM (8 ml for each 500 ml of supernatant from step 3). Wash the walls of the centrifuge bottle thoroughly, since the precipitate of bacteriophages sticks to them, especially if the bottle is old

TABLE 2.4 CsCl Solutions (100 ml) for Step Gradients Prepared in SM

Density (ρ) (g/ml)	CsCl (g)	SM (ml)	Refractive index (η)
1.45	60	85	1.3768
1.50	67	82	1.3815
1.70	95	75	1.3990

8. Extract the polyethylene glycol and cell debris from the bacteriophage suspension by adding an equal volume of chloroform and vortexing for 30 seconds. Separate the organic and aqueous phases by centrifugation at 3000*g* for 15 minutes at 4°C. Recover the aqueous phase, which contains the bacteriophage particles.

9. Measure the volume of the aqueous phase, and add 0.5 g of solid CsCl per milliliter of bacteriophage suspension. Mix gently to dissolve.

10. When the CsCl has dissolved, carefully layer the bacteriophage suspension onto CsCl step gradients that are preformed in Beckman SW41 or SW28 clear polypropylene or polycarbonate centrifuge tubes (or equivalent) (Table 2.4).

The step gradients may be made either by carefully layering solutions of decreasing density on top of one another or by layering solutions of increasing density under one another. Make a mark on the outside of the tube opposite the position of the interface between the $\rho = 1.50$ g/ml layer and the $\rho = 1.45$ g/ml layer (Figure 2.4).

11. Centrifuge at 22,000 rpm for 2 hours at 4°C in a Beckman SW41 or SW28 rotor (or equivalent).

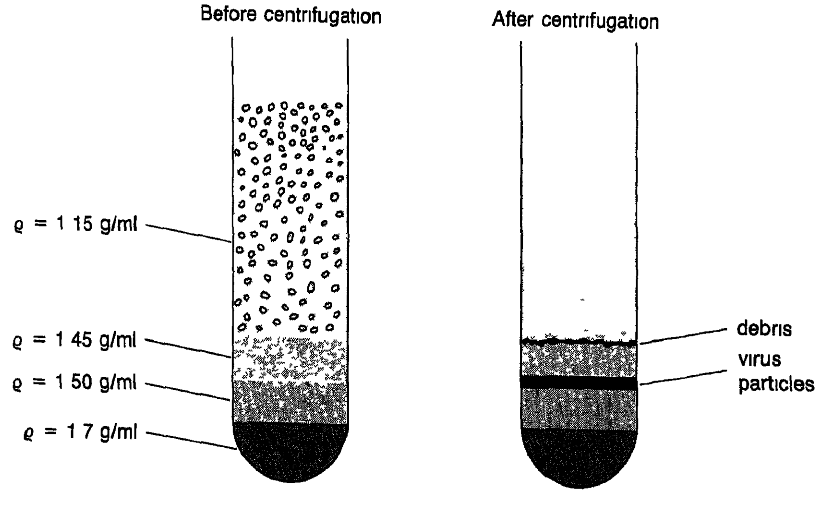


FIGURE 2.4
CsCl gradients for purifying bacteriophage λ. The bacteriophage will form a visible band at the interface between the 1.45 g/ml and 1.50 g/ml CsCl layers.

A bluish band of bacteriophage particles should be visible at the interface between the 1.45 g/ml layer and the 1.50 g/ml layer. If the yield of bacteriophage is low, placing the gradient against a black background and shining a light from above often helps to detect the band of particles.

12. Collect the bacteriophage particles by puncturing the side of the tube. Carefully wipe the outside of the tube with ethanol to remove any grease or oil, and then attach a piece of Scotch Tape to the outside of the tube, level with the band of bacteriophage particles. Using a 21-gauge needle, puncture the tube through the tape and collect the band of bacteriophage particles (Figure 2.5). *Be careful!* Keep your fingers out of the path of the needle in case you puncture the other side of the tube.

    Take care not to contaminate the bacteriophage with material from other bands that are visible in the gradient. These bands contain various types of bacterial debris and unassembled bacteriophage components.

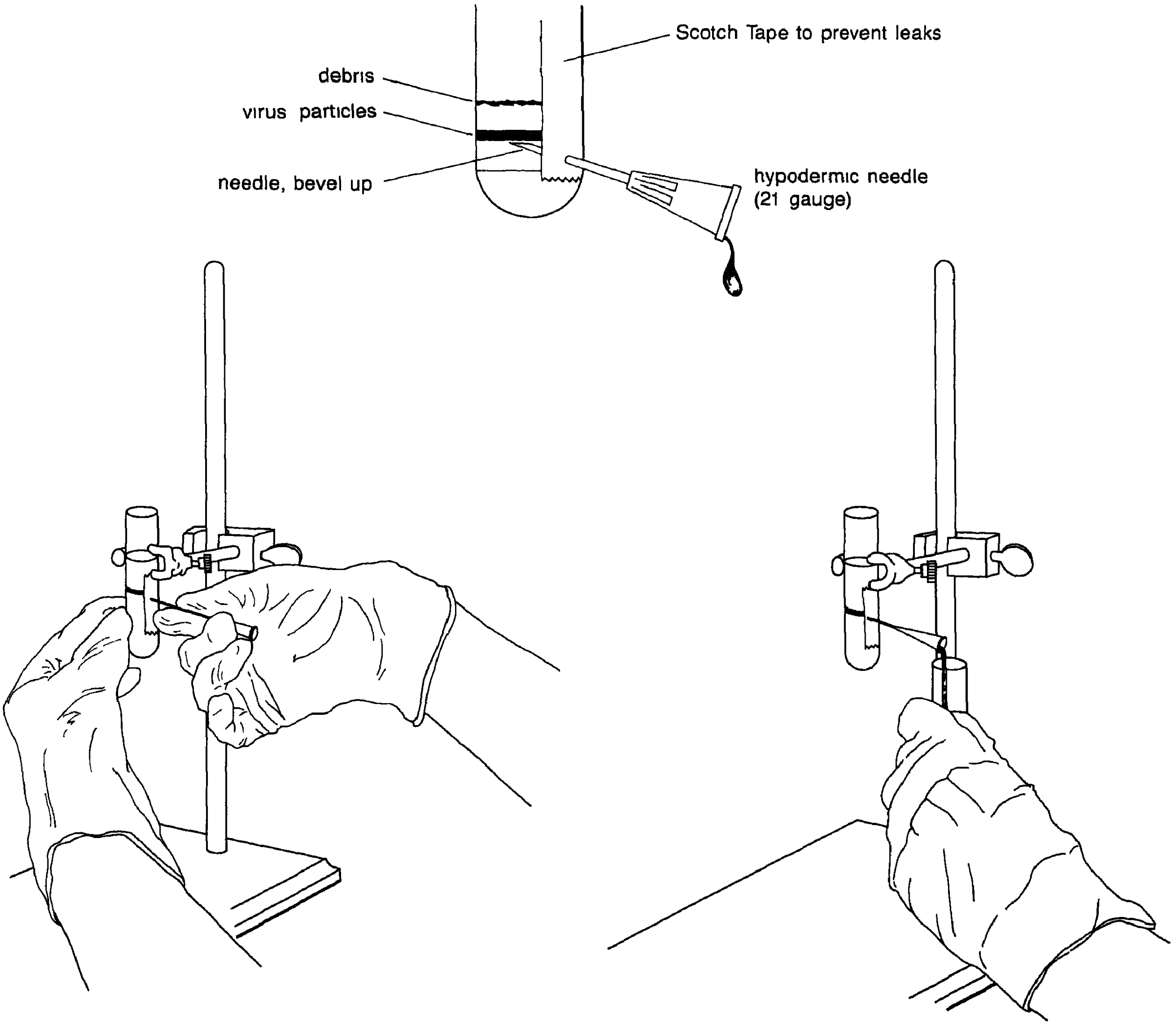


FIGURE 2.5
Collection of bacteriophage band by side puncture

Alternatively, the band can be collected from above using a micropipette or a pasteur pipette.

If a polycarbonate tube is used in step 10 or 13, the band must be collected from above

13. Place the suspension of bacteriophage particles in an ultracentrifuge tube that fits a Beckman Ti50 or SW50 1 rotor (or equivalent) and fill the tube with CsCl solution (1.5 g/ml in SM). Centrifuge at 38,000 rpm for 24 hours at 4°C (Ti50) or at 35,000 rpm for 24 hours at 4°C (SW50.1).

14. Collect the band of bacteriophage particles as described in step 12 Store the bacteriophage suspension at 4°C in the CsCl solution in a tightly capped tube.

Notes

i. Particles of bacteriophage λ are exceedingly sensitive to EDTA and other chelators, and it is essential that Mg^{++} (10–30 mM) be present at all stages of the purification to prevent disintegration of the particles

ii If the yields of purified bacteriophage are low, the number of infectious bacteriophage particles should be determined in samples taken at various stages during the purification to determine where losses are occurring

Alternative Methods for Purification of Bacteriophage λ

PELLETING BACTERIOPHAGE PARTICLES

1. Follow the "Standard Method for Purification of Bacteriophage λ" through step 8 (page 2.73), including extraction with chloroform.

2. Collect the bacteriophage particles by centrifugation at 25,000 rpm for 2 hours at 4°C in a Beckman SW28 rotor (or its equivalent).

3. Pour off the supernatant. A glassy pellet of bacteriophage particles should be visible on the bottom of the tube. Add 1–2 ml of SM to the tube, and leave overnight at 4°C, preferably on a rocking platform

4. The following morning, pipette the solution gently up and down to ensure that all of the bacteriophage particles have been resuspended. Although preparations obtained in this way are not as clean as those obtained by equilibrium gradient centrifugation, they can be a convenient source of DNA when preparing foreign DNA from bacteriophage λ recombinants for subcloning into plasmid or bacteriophage M13 vectors or when preparing bacteriophage λ arms

5. Proceed with "Extraction of Bacteriophage λ DNA" at step 5 (page 2.80).

GLYCEROL STEP GRADIENT

The following method is modified from Vande Woude et al. (1979)

1. Follow the "Standard Method for Purification of Bacteriophage λ" through step 6 (page 2.73)

2. Resuspend the bacteriophage pellet in 5–10 ml of TM per liter of original culture.

   TM

   50 mM Tris · Cl (pH 7.8)
   10 mM $MgSO_4$

   Particles of bacteriophage λ are exceedingly sensitive to EDTA and other chelators, and it is essential that Mg^{++} (10–30 mM) be present at all stages of the purification to prevent disintegration of the particles

3. Add an equal volume of chloroform to the bacteriophage suspension and vortex for 30 seconds Separate the organic and aqueous phases by centrifugation at 3000*g* for 15 minutes at 4°C. Recover the aqueous phase containing the bacteriophage particles.

4. Prepare a glycerol step gradient in a Beckman SW41 polycarbonate tube (or its equivalent) as follows.
   - a Pipette 3 ml of a solution consisting of 40% glycerol in TM into the bottom of the tube.
   - b. Carefully layer over the 40% glycerol solution 4 ml of a solution consisting of 5% glycerol in TM
   - c. Carefully layer the bacteriophage suspension onto the glycerol solution
   - d Fill the tube with TM.

5. Centrifuge at 35,000 rpm for 60 minutes at 4°C in a Beckman SW41 rotor.

6. Discard the supernatant, and resuspend the bacteriophage pellet in 1 ml of TM per liter of original culture.

7. Add pancreatic DNAase I and RNAase to final concentrations of 5 μg/ml and 1 μg/ml, respectively Digest for 30 minutes at 37°C

8. Add EDTA from a 0 5 M stock solution (pH 8 0) to a final concentration of 20 mM

9. Proceed with "Extraction of Bacteriophage λ DNA" at step 5 (page 2 80).

EQUILIBRIUM CENTRIFUGATION IN CESIUM CHLORIDE

When dealing with small-scale preparations of bacteriophage (1 liter or less), the CsCl step gradient can be omitted from the "Standard Method for Purification of Bacteriophage λ."

1. After extraction with chloroform (step 8, page 2.74), measure the volume of the aqueous phase and add 0 75 g of solid CsCl per milliliter of bacteriophage suspension Mix gently to dissolve.

2. When the CsCl has dissolved, transfer the bacteriophage suspension to an ultracentrifuge tube that fits a Beckman Ti50 or SW50 1 rotor (or equivalent) Fill the tube with SM containing 0 75 g/ml CsCl and centrifuge at 38,000 rpm for 24 hours at 4°C (Ti50) or at 35,000 rpm for 24 hours at 4°C (SW50 1)

3. Collect the band of bacteriophage particles as described in step 12, page 2.75. Store the bacteriophage suspension at 4°C in the CsCl solution in a tightly capped tube

EXTRACTION OF BACTERIOPHAGE λ DNA

1. Remove CsCl from the purified bacteriophage preparation by dialysis at room temperature for 1 hour against a 1000-fold volume of the following buffer:

   10 mM NaCl
   50 mM Tris · Cl (pH 8.0)
   10 mM $MgCl_2$

2. Transfer the dialysis sac to a fresh flask of buffer and dialyze for an additional hour.

3. Transfer the bacteriophage suspension into a centrifuge tube of a size such that only one third is full

4. Add EDTA from a 0.5 M stock solution (pH 8 0) to a final concentration of 20 mM.

5. Add pronase (self-digested) to a final concentration of 0 5 mg/ml, or add proteinase K to a final concentration of 50 μg/ml

   Self-digested pronase is prepared by dissolving powdered pronase in 10 mM Tris Cl (pH 7 5), 10 mM NaCl to a final concentration of 20 mg/ml and incubating for 1 hour at 37°C Store the self-digested pronase in small aliquots at −20°C in tightly capped tubes

6. Add SDS from a 10% w/v stock solution in water to a final concentration of 0 5% Mix by inverting the tube several times

7. Incubate for 1 hour at 37°C (pronase) or 56°C (proteinase K)

8. Cool the digestion mixture to room temperature Add an equal volume of phenol equilibrated with 50 mM Tris (pH 8.0) Mix by inverting the tube many times until a complete emulsion has formed Separate the phases by centrifugation at 3000*g* for 5 minutes at room temperature Use a wide-bore pipette to transfer the aqueous phase to a clean tube.

9. Extract the aqueous phase once with a 50:50 mixture of equilibrated phenol and chloroform

10. Recover the aqueous phase as described above, and extract once with an equal volume of chloroform.

11. Transfer the aqueous phase to a dialysis sac.

12. Dialyze overnight at 4°C against three changes of a 1000-fold volume of TE (pH 8.0)

Notes

i. The yields of bacteriophage DNA range from 500 μg to several milligrams per liter, depending on the titer of the bacteriophage in the lysed culture. When such large amounts of bacteriophage λ DNA are not required, the above methods can easily be scaled down to accommodate 50–100-ml cultures. In this case, the dialysis procedure (step 12) should be omitted and the bacteriophage DNA should be recovered by precipitation with ethanol as follows:

a. Add sodium acetate from a 3 M stock solution (pH 7.0) to a final concentration of 0.3 M. Mix well.

It is important to use a sodium acetate solution equilibrated to pH 7.0 rather than the more common pH 5.2. EDTA precipitates from solution at pH 5.2 when present at concentrations in excess of 5–10 mM.

b. Add 2 volumes of ethanol and mix.

c. Store the solution at room temperature for 30 minutes.

Usually, the bacteriophage λ DNA can be seen as a threadlike precipitate, which can be removed from the solution on the outside of a pasteur pipette. Transfer the DNA to a microfuge tube containing 1 ml of 70% ethanol. Recover the DNA by centrifugation at 12,000*g* for 2 minutes at 4°C in a microfuge. Discard the supernatant carefully, and allow the pellet of DNA to dry at room temperature. Redissolve the DNA in an appropriate volume of TE (pH 7.6).

ii. A particle of bacteriophage λ contains $\sim 5 \times 10^{-11}$ μg of DNA.

Cloning in Bacteriophage λ

Bacteriophage λ has many uses as a cloning vehicle, ranging from subcloning of foreign DNA sequences propagated in vectors with larger capacity (e.g., cosmids) to construction of complex cDNA or genomic DNA libraries. Cloning in bacteriophage λ involves five steps:

1. Preparation of vector DNA by cleavage with appropriate restriction enzyme(s).
2. Ligation of the digested vector with fragments of foreign DNA having termini compatible with those of the vector.
3. Packaging of the ligated DNA into bacteriophage particles that can form plaques on appropriate bacterial hosts.
4. Identification of recombinant bacteriophages carrying the desired sequences of foreign DNA.
5. Plaque purification of selected recombinant bacteriophages, followed by analysis of the foreign DNA sequences.

PREPARATION OF VECTOR DNA

In some cases, bacteriophage λ DNA can be prepared for cloning by simple digestion with restriction enzymes. For example, when using vectors that have been designed to allow genetic selection of bacteriophages carrying sequences of foreign DNA (e.g., the EMBL series, λ2001, λDASH, or λgt10), it is not always necessary to take steps to minimize the formation of nonrecombinant bacteriophages. In addition, for certain applications (e.g., cloning a segment of purified DNA), the presence of a high proportion of nonrecombinant bacteriophages is not a significant detriment, since the desired recombinant can be easily identified by plaque hybridization. However, more complex cloning problems require the use of biochemical or physical methods to minimize the number of nonrecombinant bacteriophages. These include physical separation of the stuffer fragment from the vector arms, treatment of the digested vector DNA with alkaline phosphatase to reduce the possibility of regenerating vector DNA by ligation of the left and right arms, and digestion of vectors with combinations of restriction enzymes that will destroy the ability of the stuffer fragment to reinsert into the arms.

The procedures in this section describe the methods used to prepare and test bacteriophage λ vectors for cloning. Detailed procedures for the preparation and ligation of foreign DNA for cDNA or genomic DNA cloning are given in Chapters 8 and 9, respectively.

Digestion of Bacteriophage λ DNA with Restriction Enzymes

1. Mix 25–50 μg of bacteriophage λ DNA with TE (pH 8 0) to give a final volume of 170 μl
2. Add 20 μl of the appropriate restriction enzyme buffer. Remove two aliquots (0 2 μg each) of the undigested DNA and store on ice.
3. Add a threefold excess (75–150 units) of the appropriate restriction enzyme and incubate for 1 hour at the optimal temperature recommended by the manufacturer
4. Cool the reaction to 0°C on ice. Remove an aliquot (0.2 μg) Incubate this aliquot and one of the two aliquots of undigested DNA (step 2 above) for 10 minutes at 68°C to disrupt the cohesive termini of the bacteriophage DNA, and analyze the resulting DNA preparations by electrophoresis through a 0.5% agarose gel. If digestion is complete, no DNA will be visible migrating at the position of the undigested control DNA; instead, two or more (depending on the number of cleavage sites in the vector) smaller fragments will be seen The number and yield of these smaller fragments should be examined carefully to ensure that no partial digestion products are present
5. If digestion is incomplete, warm the reaction to the appropriate temperature, add more restriction enzyme (50–100 units), and continue the incubation at the optimal temperature recommended by the manufacturer.
6. When digestion is complete, add EDTA to a final concentration of 5 mM, and extract the digestion mixture once with phenol:chloroform and once with chloroform
7. Recover the DNA from the aqueous phase by precipitation with ethanol (see Note i, a–c, page 2 81) Redissolve the DNA in 100 μl of TE (pH 8.0), and determine its exact concentration by measuring its absorbance at 260 nm (see Appendix E).
8. Remove an aliquot of DNA (1 μg), and test whether it can be efficiently ligated as follows

   a Add water to bring the volume to 17 μl

   b Add 2 μl of 10 × ligation buffer and 1 μl of 10 mM ATP.

10 × Ligation buffer

0.5 M Tris·Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

c. Remove 5 μl of the mixture prepared in step b and store on ice

d Add 0.2–0.5 Weiss unit of bacteriophage T4 DNA ligase to the remainder of the mixture, and incubate the reaction for 2 hours at 16°C.

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al 1968) One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C One Weiss unit corresponds to 0 2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs) 0 015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units

e. Package 2 μl (0.1 μg) of the ligated and unligated samples and 0 1 μg of the undigested vector DNA from step 2 (See page 2 107 for this protocol.) Titrate each of the packaging reactions

The packaging efficiency of the digested vector should increase by nearly three orders of magnitude after ligation The packaging efficiency of the ligated sample should be approximately 10% of that of undigested vector DNA

Note

The background of plaques obtained after cleavage of bacteriophage λ vectors at a single restriction site is often unacceptably high because of the accumulation of mutants in the original population that cannot be cleaved with the restriction enzyme This problem can be avoided if the bacteriophage λ vector is plaque-purified frequently.

Purification of Bacteriophage λ Arms

Many of the bacteriophage λ vectors that are most useful in the construction of cDNA or genomic DNA libraries require the middle stuffer segments of their genomes to be removed to accommodate large segments of foreign DNA. This process is generally referred to as "preparation of arms." Depending on the vector used, any of several approaches are possible. Two methods of purifying arms by density gradient centrifugation are described below. Alternatively, arms can be purified by preparative electrophoresis through 0.5% agarose gels (see Chapter 6). In general, however, the yield of arms obtained by this technique is much lower than that obtained by density gradient centrifugation. Purified arms of several of the most popular vectors (e.g., EMBL3 and 4, λgt10, λgt11) are available from commercial sources. However, it is less expensive to make your own arms if they are to be used regularly for the construction of libraries.

CENTRIFUGATION THROUGH SUCROSE DENSITY GRADIENTS

This method (Maniatis et al. 1978) can be used to prepare the arms of any bacteriophage λ vector DNA.

1. Digest and analyze 60 μg or more of the bacteriophage λ vector DNA as described in steps 1–7 on page 2.83. After step 7 of the procedure, adjust the concentration of the DNA with TE (pH 8.0) to 150 μg/ml or greater. Set aside a 0.2-μg aliquot for use as a gel marker in step 6.

It is sometimes possible to digest the vector DNA with a restriction enzyme that cleaves within the stuffer fragment but not the arms. The aims of this strategy are (a) to reduce the size of the stuffer fragment(s) and thereby improve the separation of the stuffer from the arms and (b) to produce termini that are incompatible with those of the arms.

The digested DNA may be stored at −20°C until you are ready to proceed to step 2.

2. Add $MgCl_2$ to 0.01 M, and incubate for 1 hour at 42°C to allow the cohesive termini of bacteriophage λ DNA to reanneal. Analyze an aliquot (0.2 μg) by electrophoresis through a 0.5% agarose gel to determine whether annealing has occurred. Use as markers 0.2 μg of intact bacteriophage λ DNA (from step 2, page 2.83) and a 0.2-μg aliquot of the annealed DNA that has been heated to 68°C for 10 minutes.

Annealing of the cohesive termini causes the arms to increase in size. The annealed arms migrate through the 0.5% agarose gel at a rate that is usually indistinguishable from that of intact bacteriophage λ DNA. Do not run the analytical gels at high voltage or in electrophoresis buffers of high electrical resistance; overheating will melt the cohesive termini of bacteriophage λ DNA during electrophoresis.

An alternative procedure is to ligate the cohesive termini of the vector DNA before digesting it with restriction enzyme(s). Incubate the DNA for 1 hour at 42°C in 170 μl of 0.1 M Tris Cl (pH 7.6), 10 mM $MgCl_2$. Add 20 μl of 10× ligation buffer (see page 2.83), and 0.2–0.5 Weiss unit of bacteriophage T4 DNA ligase per microgram of DNA, and incubate for 1–2 hours at 16°C. (For definition of Weiss unit, see note to d, page 2.84.) At this point, the DNA will have formed long concatemers and will be more sensitive to shearing Handle it carefully! After gentle extraction with phenol.chloroform (shaking the minimum amount to form an emulsion), recover the DNA by precipitation with ethanol (see page 2 81), resuspend it in the appropriate buffer, and digest it with restriction enzyme(s).

3. Prepare one or more 38-ml sucrose (10–40% w/v) gradients in polypropylene tubes (Beckman SW28 or equivalent). Each gradient can accommodate 60–75 μg of digested bacteriophage λ DNA The sucrose solutions are made in a buffer containing:

   1 M NaCl
   20 mM Tris · Cl (pH 8.0)
   5 mM EDTA (pH 8 0)

4. Load onto each gradient no more than 75 μg of annealed, digested bacteriophage λ DNA in a volume of 500 μl or less. Loading more DNA than this can cause the gradient to be overloaded and can lead to poor separation of the stuffer fragments from the arms Centrifuge at 26,000 rpm for 24 hours at 15°C in a Beckman SW28 rotor (or its equivalent).

5. Collect 0.5-ml fractions through a 21-gauge needle inserted through the bottom of the centrifuge tube.

6. Dilute 15 μl of every third fraction with 35 μl of H_2O. Add 8 μl of gel-loading buffer I (see Appendix B), heat the samples to 68°C for 5 minutes, and analyze them by electrophoresis through a thick 0 5% agarose gel. Use as markers intact bacteriophage λ DNA and the aliquot of digested DNA set aside in step 1 Adjust the sucrose and salt concentrations of the markers to match those of the samples, otherwise, their electrophoretic mobilities will not be comparable

   After photographing the gel (see Chapter 6, page 6 19), locate and pool the fractions that contain the annealed arms (Figure 2 6) Be careful not to include fractions that are visibly contaminated with undigested bacteriophage λ DNA or that contain significant quantities of unannealed left or right arms or stuffer fragment(s).

7. Dialyze the pooled fractions against a 1000-fold excess of TE (pH 8 0) at 4°C for 12–16 hours, with at least one change of buffer. Be sure to allow for a two- to threefold increase in volume during dialysis. Extract the sample several times with 2-butanol (see Appendix E) to reduce the volume to less than 3 ml

   Alternatively, if the pooled sample is small, the DNA can be precipitated with ethanol without prior dialysis by diluting the sample with TE (pH 7 6) so that the concentration of sucrose is reduced to about 10%

8. Recover the DNA by precipitation with ethanol (see page 2 81)

9. Dissolve the DNA in TE (pH 7.6) at an approximate concentration of 300–500 μg/ml.

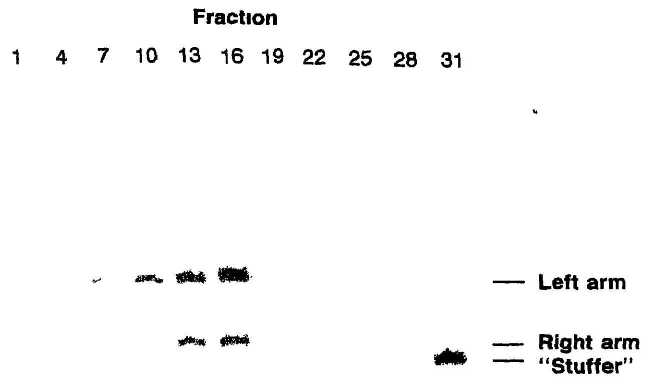


FIGURE 2.6
Preparation of the arms of bacteriophage λ DNA by sucrose gradient centrifugation. In this experiment, the DNA of bacteriophage λ vector Charon 28 was digested with *Bam*HI, annealed, and centrifuged through a 10–40% sucrose gradient, which was then fractionated as described in the text. Aliquots of every third fraction were heated briefly to 68°C and analyzed by electrophoresis through a 0.5% agarose gel. The positions of the left arm (23.5 kb), right arm (9 kb), and "stuffer" fragments (6.5 kb) are indicated. Fractions 1–16 containing the annealed arms were pooled.

10. Measure the exact concentration of the DNA spectrophotometrically (see Appendix E), and analyze an aliquot by electrophoresis through a 0.5% agarose gel to assess its purity. Store the DNA at −20°C in aliquots containing 1–5 μg.

Note

Ethidium bromide, at a concentration of 2 μg/ml, can be included in sucrose density gradients. The positions of the different species of DNA within the gradient can then be determined visually. With practice, it is possible to pool those fractions that contain the annealed arms without prior analysis by agarose gel electrophoresis. If ethidium bromide is used in this way, the purified arms should be extracted three times with isoamyl alcohol to remove the dye (see Appendix B).

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when you are working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

CENTRIFUGATION THROUGH SODIUM CHLORIDE GRADIENTS

This procedure is quicker than centrifugation through sucrose density gradients. However, more care must be taken in preparing sodium chloride gradients because of the lower viscosity of the solutions.

1. Digest and analyze 60 μg or more of the bacteriophage λ vector DNA as described in steps 1–7 on page 2.83. After step 7 of the procedure, adjust the concentration of the DNA with TE (pH 8.0) to 250–500 μg/ml and reanneal the arms as described in step 2, page 2.85.

2. Prepare one or more 12.5-ml continuous gradients of NaCl (5–25% w/v) in ultracentrifuge tubes (Beckman SW41 or equivalent) using a commercial gradient maker. Each gradient can accommodate 50–100 μg of digested bacteriophage λ DNA.

   The NaCl solutions contain 10 mM Tris·Cl (pH 8.0), 10 mM EDTA (pH 8.0). Prepare the gradients in a cold room at a low flow rate (1–2 ml/minute) using a glass capillary to deliver the solution to the surface of the liquid. This minimizes mixing.

3. Load onto each gradient 50–100 μg of annealed, digested bacteriophage λ DNA in a volume of 200 μl or less. Centrifuge at 37,000 rpm for 4.5 hours at 4°C in a Beckman SW41 rotor (or its equivalent).

4. Collect approximately 0.25-ml fractions from the bottom of each tube using a gradient-fractionating device. Be careful! The gradients drip very rapidly after the tubes have been punctured.

5. Dilute 5 μl of every third fraction with 30 μl of H_2O. Add 4 μl of gel-loading buffer I (see Appendix B), heat the samples to 68°C for 5 minutes, and analyze the samples by electrophoresis through a thick 0.5% agarose gel. Use as markers intact bacteriophage λ DNA and bacteriophage λ DNA that has been digested with the appropriate restriction enzyme. Adjust the salt concentrations of the markers to match those of the samples, otherwise, their electrophoretic mobilities will not be comparable.

   After photographing the gel (see Chapter 6, page 6.19), locate and pool the fractions that contain the annealed arms. Be careful not to include fractions that are visibly contaminated with undigested bacteriophage λ DNA or that contain significant quantities of unannealed left or right arms or stuffer fragment(s).

6. Add an equal volume of water to the pooled fractions and mix well. Recover the DNA by precipitation with ethanol (see page 2.81). Redissolve the DNA in TE (pH 7.6) at an approximate concentration of 300–500 μg/ml.

7. Measure the exact concentration of the DNA spectrophotometrically (see Appendix E), and analyze an aliquot by electrophoresis through a 0.5% agarose gel to assess its purity. Store the DNA at −20°C in aliquots containing 1–5 μg.

Note

Ethidium bromide, at a concentration of 2 μg/ml, can be included in sodium chloride gradients. The positions of the different species of DNA within the gradient can then be determined visually With practice, it is possible to pool those fractions that contain the annealed arms without prior analysis by agarose gel electrophoresis. If ethidium bromide is used in this way, the purified arms should be extracted three times with isoamyl alcohol to remove the dye (see Appendix B)

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when you are working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

Preparation of Vectors Treated with Alkaline Phosphatase

Removal of the 5′-phosphate groups from the termini of bacteriophage λ arms can effectively prevent self-ligation and reduce the background of nonrecombinant bacteriophages. This method should be used when the vector contains a single site for cloning (e.g., λgt10, λgt11, λgt18–23, λZAP, or λORF8) or when a stuffer fragment cannot be removed by physical procedures. It is very important that the cohesive termini of the vector be reannealed and ligated before treatment with phosphatase; otherwise, concatemerization will be inhibited and the packaging efficiency of the DNA will be reduced significantly.

1. Ligate the cohesive termini of undigested vector DNA as described in the second note to step 2, page 2.85, and digest the ligated DNA with the appropriate restriction enzyme (steps 1–3, page 2.83). Test the efficiency of packaging of intact bacteriophage λ DNA (0.01 μg) and annealed/ligated DNA (0.1 μg) before and after digestion with the restriction enzyme (see page 2.107 for the packaging protocol). If digestion is complete, the packaging efficiency should be reduced by at least three orders of magnitude. Purify the digested DNA by extraction with phenol:chloroform and chloroform followed by precipitation with ethanol (see Appendix E).

2. Resuspend the DNA at a concentration of 100 μg/ml in 10 mM Tris·Cl (pH 8.3), and store an aliquot (0.5 μg) on ice. Treat the remainder of the DNA with an excess of calf intestinal alkaline phosphatase (CIP) for 1 hour at 37°C as follows:

   a. Add 0.1 volume of 10 × CIP dephosphorylation buffer and 0.01 unit of CIP for every 10 μg of bacteriophage λ DNA.

      10 × CIP dephosphorylation buffer

      10 mM $ZnCl_2$
      10 mM $MgCl_2$
      100 mM Tris·Cl (pH 8.3)

   b. Mix, and incubate for 30 minutes at 37°C. Then add a second aliquot of CIP and continue incubation for an additional 30 minutes at 37°C.

      If the arms carry blunt or recessed 5′ termini, the second half of the incubation should be carried out at 55°C.

      Approximately 0.01 unit of CIP is needed to remove the terminal phosphates from 1 pmole of 5′ termini of DNA (1 pmole of 5′ termini of a 40-kb linear DNA is 16 μg).

3. Add SDS and EDTA (pH 8.0) to final concentrations of 0.5% and 5 mM, respectively. Mix well, and add proteinase K to a final concentration of 100 μg/ml. Incubate for 30 minutes at 56°C.

Proteinase K is used to digest the CIP, which must be completely removed if the subsequent ligation reactions are to work efficiently. An alternative method to inactivate the CIP is to heat the reaction (at the end of step 2) to 65°C for 1 hour (or 75°C for 10 minutes) in the presence of 5 mM EDTA and then to extract once with phenol:chloroform.

4. Cool the reaction to room temperature, and purify the bacteriophage λ DNA by extracting once with phenol·chloroform and once with chloroform alone. Add 0.1 volume of 3 M sodium acetate (pH 7.0). Mix well, and add 2 volumes of ethanol. Mix well, and store at 0°C for 15 minutes. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol at 4°C and recentrifuge.

5. Redissolve the DNA in TE (pH 7.6) at a concentration of 300–500 μg/ml. Store the dephosphorylated DNA at −20°C in aliquots containing 1–5 μg.

6. Measure the efficiency of the phosphatase treatment by ligating a portion (0.5 μg) of the digested vector before and after treatment with phosphatase (see discussion of trial ligations on page 2.94). Package the DNA into bacteriophage particles (see page 2.107 for protocol), and titrate the infectivity. Phosphatase treatment should reduce ligation and the efficiency of packaging of the arms by two to three orders of magnitude.

Notes

i The pH of the 3 M sodium acetate solution is 7.0 rather than the usual 5.2. At acid pH, EDTA precipitates from solution if its concentration exceeds 5–10 mM.

ii Until recently, CIP was supplied as a suspension in a slurry of ammonium sulfate. However, it may now be obtained as a stabilized solution (in 30 mM triethanolamine buffer [pH 7.6], 3 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$). It should be stored undiluted in this buffer (not frozen).

Digestion of Bacteriophage λ Vectors with Two Restriction Enzymes

Vectors such as the EMBL series, λ2001, λDASH, and Charon 34, 35, and 40 contain a series of restriction sites, arranged in opposite orientations, in polycloning sites at each end of the central stuffer fragment (Frischauf et al 1983). In EMBL3A, for example, the order of restriction sites in the left polycloning site is *Sal*I–*Bam*HI–*Eco*RI and the order in the right polycloning site is *Eco*RI–*Bam*HI–*Sal*I. Digestion of such a vector with both *Bam*HI and *Eco*RI yields left and right arms that carry *Bam*HI termini, a stuffer fragment carrying *Eco*RI termini, and short segments of the polycloning sites carrying *Eco*RI and *Bam*HI termini. These short segments can easily be removed by precipitation with isopropanol (see Appendix E) or by centrifugation through a spun column of Sepharose CL-4B (see Appendix E) The arms are then unable to ligate to the stuffer fragment because *Eco*RI and *Bam*HI termini are incompatible In this way, it is possible to obtain a decrease of two orders of magnitude in the number of nonrecombinant bacteriophages When this "biochemical selection" is used in combination with a genetic selection (e g , Spi), the number of nonrecombinant bacteriophages can be reduced by an additional two to three orders of magnitude This combined approach can be used with the EMBL bacteriophages, λ2001, and λDASH

1. Digest the vector DNA with one of the two appropriate restriction enzymes as described in steps 1–3, page 2 83. Use gel electrophoresis to check that digestion is complete

2. Purify the DNA by extracting twice with phenol chloroform and once with chloroform alone

3. Recover the DNA from the aqueous phase by precipitation with ethanol Redissolve the DNA in TE (pH 7 6) at a concentration of 100–150 μg/ml Store an aliquot (0 5 μg) at −20°C

4. Digest the DNA with the second restriction enzyme Use a fourfold excess of enzyme, and incubate the reaction for 4 hours

   The efficiency of the second digestion can be monitored in one of two ways
   - The series of steps outlined below (steps 5–7) can be completed, and the efficiency of packaging of the final preparation can then be measured before and after ligation If the number of nonrecombinant bacteriophages is unacceptably high, the digestion and analysis can be repeated
   - Alternatively, a small fraction of the product of the first digestion can be end-labeled (see Chapter 1, page 1 56), the radiolabel can then be used to monitor digestion by the second restriction enzyme If this method is used, it is important to ligate the cohesive termini of the vector DNA before digestion with the first restriction enzyme (see second note to step 2, page 2 85) This ligation prevents incorporation of significant amounts of radiolabel into the cohesive termini After end-labeling, add a small quantity of the radioactive DNA (0 01–0 1 μg) to the remainder of the vector Digestion with the second restriction enzyme should result in quantitative movement of the label from the large stuffer fragment and arms into the small polycloning site, this can be readily monitored by gel electrophoresis and autoradiography

5. Purify the DNA by extracting twice with phenol:chloroform and once with chloroform alone.

6. Recover the DNA from the aqueous phase by precipitation with ethanol (see Appendix E). Redissolve the DNA in TE (pH 7.6) at a concentration of 300–500 μg/ml.

7. To determine the effectiveness of the procedure, set up trial ligation reactions (as discussed on page 2.94) using 0.5 μg of the vector digested with only the first enzyme (the aliquot set aside at the end of step 3 above) and 0.5 μg of the final preparation. Package equivalent amounts of DNA (0.1 μg) from each ligation mixture, and titrate the infectivity of the resulting bacteriophage particles. The efficiency of packaging of the doubly digested vector should be two to three orders of magnitude less than that of the vector DNA digested with only one restriction enzyme.

Ligation of Bacteriophage λ Arms to Fragments of Foreign DNA

There are two important parameters to consider in ligating bacteriophage λ arms to segments of foreign DNA: the ratio of arms to potential inserts and the concentration of each DNA species in the reaction mixture. Optimum values for both of these parameters can be estimated from theoretical considerations (see Chapter 1, pages 1.63–1.67). By necessity, however, such calculations assume that all the DNA molecules in the ligation reaction are perfect. Since this is rarely the case, it is advisable to carry out pilot reactions to check the efficiency of each new preparation of arms and potential inserts.

Typically, trial ligations contain approximately 0.5–1.0 μg of bacteriophage λ arms and a range of concentrations of foreign DNA. The *molar* ratio of arms:potential inserts in the test ligations should range from 0.25:1 to 8:1 (see Table 2.5) and the volume of the ligation mixture should be as small as possible (10 μl or less). It is essential to include controls that contain bacteriophage λ arms but no insert and insert but no bacteriophage λ arms. Once assembled, the ligation reactions are usually incubated at 16°C for 4–6 hours. If desired, a sample of each trial ligation can then be analyzed by electrophoresis through 0.5% agarose gels. If the ligation reactions have been successful, almost all of the DNA should be at least as large as intact bacteriophage λ DNA.

A sample (25%) of each ligation reaction should be used as the substrate for an in vitro packaging reaction. The ligation containing the optimum ratio of arms:inserts should yield at least 10^6–10^7 recombinants per microgram of bacteriophage λ arms when a commercial packaging mixture such as Gigapack Gold (Stratagene) is used.

TABLE 2.5 Amounts of Potential Insert DNA Used in Trial Ligations of Bacteriophage λ Arms

Size (kb) of potential insert DNA	Amount of potential insert DNA to be used in trial ligations
2–4	6–200 ng
4–8	12–400 ng
8–12	24–600 ng
12–16	36–800 ng
16–20	48 ng–1.0 μg
20–24	60 ng–1.2 μg

To determine the ratio of arms:potential inserts that yields the maximum number of recombinant bacteriophages, a series of trial ligations should be set up that contain a constant amount of arms and different amounts of potential insert. The amounts of inserts recommended in Table 2.5 have been calculated on the assumption that the ligation mixtures will contain 1 μg of bacteriophage λ arms, 40 kb in size. In ligation mixtures containing the lowest recommended amount of potential insert, the bacteriophage λ arms will be present in eightfold molar excess; in mixtures containing the greatest amount of potential insert, the insert will be present in fourfold molar excess.

CKAGING OF BACTERIOPHAGE λ DNA IN VITRO

Packaging of bacteriophage λ DNA in vitro was initially developed by Becker and Gold (1975) using mixtures of extracts prepared from bacteria infected with bacteriophage λ mutant in genes required for assembly of bacteriophage particles. The procedure has been improved and modified in a number of laboratories to the point where efficiencies of 10^8 pfu/μg of intact bacteriophage λ DNA can now be reproducibly attained Approximately 0.05–0.5% of the DNA molecules present in the reaction can be packaged into infectious virions. A diagrammatic representation of the various stages of packaging of bacteriophage λ DNA and the stages at which various gene products are involved in the process is shown in Figure 2.3 (see page 2.7).

The E *protein is the major component of the bacteriophage head and is* required for the assembly of the earliest identifiable precursor. Bacteriophages mutant in the *E* gene accumulate all of the components of the viral capsid The D protein is localized on the outside of the bacteriophage head and is involved in the coupled process of insertion of bacteriophage λ DNA into the "prehead" precursor and the subsequent maturation of the head. Bacteriophages mutant in the *D* gene accumulate the immature prehead but do not allow insertion of bacteriophage λ DNA into the head. The A protein is involved in the insertion of bacteriophage λ DNA into the bacteriophage prehead and cleavage of the concatenated precursor DNA at the *cos* sites. Bacteriophages mutant in the *A* gene also accumulate empty preheads Complementing extracts have been prepared from cells infected with A^- and E^- or D^- and E^- strains, alternatively, extracts prepared from cells infected with A^- mutants can be complemented by the addition of *purified wild-type A protein*

Extracts are usually prepared from cells containing bacteriophage λ lysogens of the appropriate genotype (amber mutations in the *A, D,* or *E* gene) The lysogens also carry one or more of the following mutations:

- *c*I*ts*857 or *imm*434 *c*I*ts*, which specify a temperature-sensitive bacteriophage λ repressor molecule This mutation causes bacteriophage λ DNA to be maintained in the lysogenic state when the host bacteria are grown at 32°C; lytic growth of the bacteriophage is induced by transiently raising the temperature to 45°C to inactivate the repressor specified by the *c*I gene

- *S*am7, an amber mutation in the bacteriophage *S* gene that is required for cell lysis This mutation causes capsid components to accumulate within bacterial cells that do not contain a *supF* mutation for 2–3 hours following induction of the *c*I*ts*857 lysogen

- *b*-region deletion (*b*2 or *b*1007), a deletion in the bacteriophage genome that removes the bacteriophage λ DNA attachment site (*att*). This mutation reduces, but does not entirely eliminate, the packaging of endogenous bacteriophage λ DNA molecules in extracts made from the induced cells.

- *red*3 (in bacteriophage λ) and *recA* (in *E coli*), mutations that inactivate the generalized recombination systems of bacteriophage λ and the host, thereby minimizing recombination between the endogenous bacteriophage λ DNA in the packaging extract and the exogenously added recombinant genomes.

Maintenance and Testing of Lysogens of Bacteriophage λ

Because successful preparation of packaging extracts is dependent on the presence of specific mutations in the bacteriophage λ genome, considerable care should be exercised in growing and maintaining the lysogens used to prepare the extracts We recommend taking the following precautions

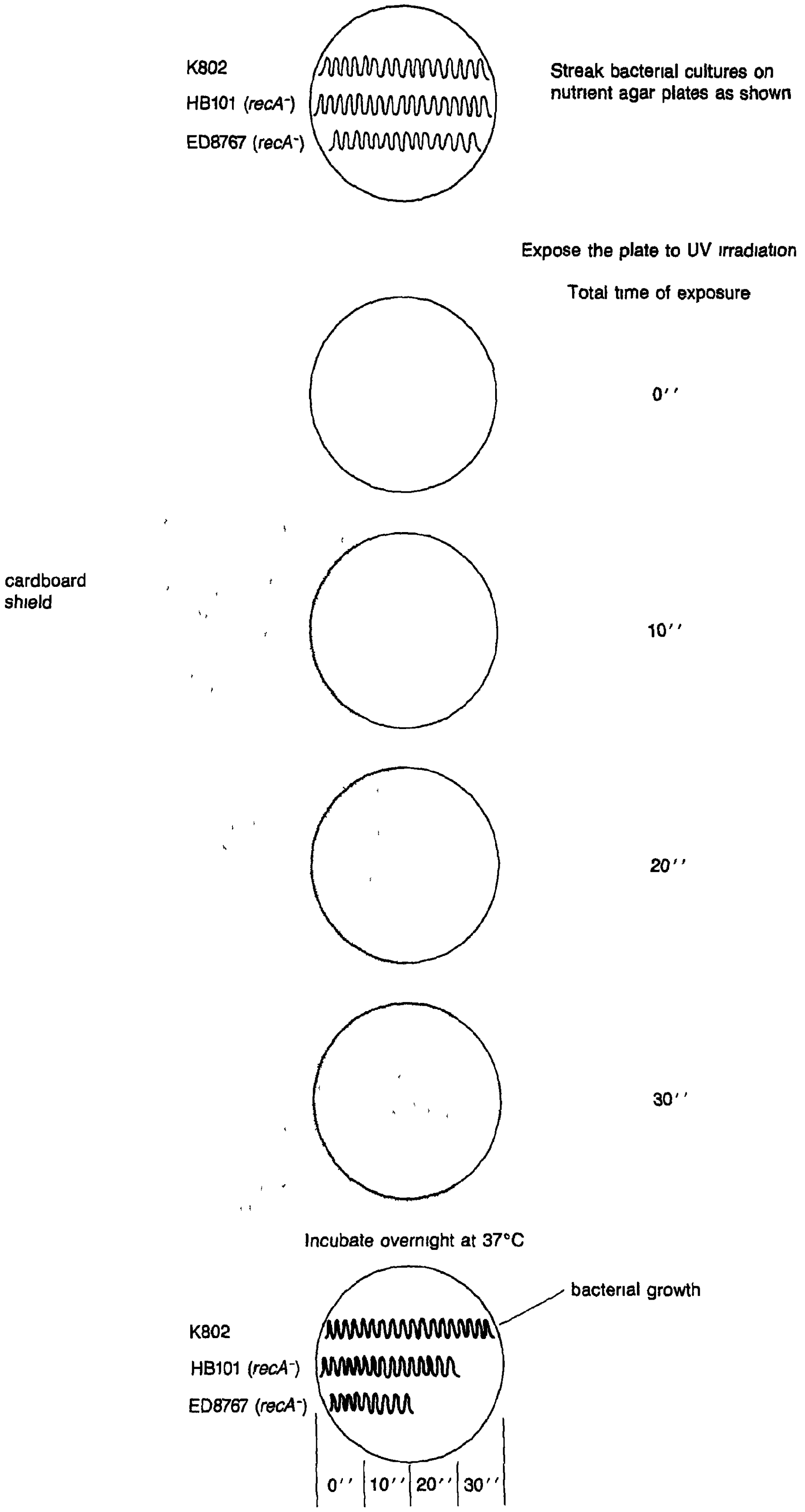
1. Master stocks of *E. coli* lysogens should be stored in glycerol at −70°C as described in Appendix A. Extracts should be made from cultures prepared directly from these master stocks as described on pages 2 100–2 103 and 2.105–2 106.
2. Before growing large-scale cultures, check for the presence of the mutation that renders the bacteriophage *c*I gene product temperature-sensitive by streaking from the master stocks of the lysogens onto LB agar plates (two plates for each bacterial strain) Incubate one plate of each strain at 32°C and the other at 45°C. The bacteria should grow only on the plates incubated at 32°C Pick a single colony of each strain from the plates incubated at 32°C, and grow small overnight cultures at 32°C Test these cultures once again by plating aliquots at 32°C and 45°C as described below. If the bacteria grow only on the plates incubated at 32°C, the remainder of the small overnight cultures may be used as inocula for larger cultures from which extracts are prepared.

Pick small, rather than large, colonies since the *recA*$^-$ mutation present in the BHB strains used to prepare extracts in Protocol I causes the bacteria to grow slowly Revertants and pseudorevertants therefore have a selective advantage and give rise to larger colonies If necessary, the presence or absence of the *recA* function in the lysogens can be tested as follows

i Streak the strain being tested, a known *recA*$^-$ strain, and a wild-type *E coli* strain onto a plate as shown in Figure 2 7 (top)

ii Use a piece of cardboard to cover about one quarter of each streak Expose the remaining portion of each streak for 10 seconds to a hand-held ultraviolet light Move the cardboard so that only half of each streak is now visible, and repeat the exposure to ultraviolet light Move the cardboard once again so that three quarters of the streak is covered, and repeat the exposure to ultraviolet light Following overnight growth, the plate should look like the one illustrated in Figure 2 7 (bottom)

Mutations in the *recA* gene prevent repair of damage induced in DNA by ultraviolet light, so that irradiated *recA*$^-$ cells do not grow Different *recA*$^-$ mutants are killed by different amounts of ultraviolet irradiation, but all are far more sensitive than *recA*$^+$ strains


Streak bacterial cultures on nutrient agar plates as shown

Expose the plate to UV irradiation

Total time of exposure

Incubate overnight at 37°C

FIGURE 2.7

Preparation of Packaging Extracts and Packaging of Bacteriophage λ DNA In Vitro

Packaging extracts are available at reasonable cost from commercial sources These work extremely well and have largely obviated the need for individual research laboratories to prepare their own extracts. The methods given below will be of use primarily to workers who use packaging extracts in such large quantity that it would be improvident to purchase them

Packaging extracts are usually prepared by growing the appropriate lysogenic bacteria to mid-log phase at 32°C, inducing lytic functions by inactivating the *c*I repressor protein by raising the temperature to 45°C for 15 minutes, and then growing the cultures for an additional 2–3 hours at 38–39°C to allow packaging components to accumulate Cell extracts are then prepared.

Of the many protocols available for the preparation of packaging extracts, the two presented below are the simplest to perform and are highly efficient Protocol I is representative of the classical type of packaging in that two lysogens that provide complementing components of the packaging reaction are grown and induced separately Extracts of each culture are prepared and blended into a mixture that contains all of the components necessary for packaging of bacteriophage λ DNA. Neither lysogen alone is capable of packaging exogenously added DNA The protocol is highly reproducible and simple, and it results in packaging mixtures that are efficient and free from background (when assayed on hosts that are nonsuppressing). Two alternative, but slightly more complicated, procedures for preparing this type of binary packaging extract are also available. The protocol described by Sternberg et al. (1977) is efficient, but it involves two different extracts, that described by Faber et al (1978) depends on the purification of the bacteriophage λ A protein and the preparation of two extracts

Protocol II (Rosenberg et al 1985; Rosenberg 1987) differs from the classical procedure in two important respects. (1) Only a single lysogenic strain is used, and only one extract is prepared (2) The lysogenic bacteria are a strain of *E coli* C.

A single lysogen can be used to prepare packaging extracts for two reasons. First, the bacteriophage λ genome lysogenic in the strain codes for all of the proteins needed for packaging. Second, the *cos* site of the prophage has been deleted These features work together in the following manner Induction of the lysogen results in the intracellular accumulation of all protein components needed for packaging, and complete preheads are formed. However, the next steps in the packaging process are the recognition of the *cos* sites on concatenated bacteriophage λ DNA by the bacteriophage A protein and the insertion of the bacteriophage λ genome into the prehead The lack of the *cos* site in the prophage DNA prevents this step from occurring, and packaging is thus effectively halted at the prehead stage, even though all necessary components that are used later in the process are present. Exogenous DNA with an active *cos* site is inserted into the prehead, and the packaging process then leads to the production of an infectious bacteriophage particle. Extracts made in this way usually have a lower background of plaques than the classical binary mixtures, because the deletion of the *cos* site blocks packaging of endogenous bacteriophage λ DNA more completely than does the deletion of the *b* region.

E. coli C was chosen as the host for the lysogen to lessen the probability of recombination between cryptic bacteriophage λ prophages, which are known to be present in the genome of *E coli* K, and the *cos*-deleted prophage. Furthermore, *E coli* C lacks the *Eco*K restriction system (Rosenberg 1985). This system, like other restriction systems, cuts unmodified DNA in a sequence-specific manner and is also functional under the conditions of an in vitro packaging reaction Thus, extracts prepared from cells of *E. coli* K have the potential to select against DNA that contains an unmodified *Eco*K recognition site Because eukaryotic DNA used to construct libraries will not be protected from cleavage, clones that by chance contain an *Eco*K recognition site may be lost from the population during packaging. Reconstruction experiments show that bacteriophage λ DNA carrying an *Eco*K recognition site is packaged two- to sevenfold less efficiently in extracts derived from *E. coli* K than in extracts prepared from *E. coli* C (Rosenberg 1985)

PROTOCOL I: PREPARATION OF PACKAGING EXTRACTS FROM TWO LYSOGENS

In this procedure, two separate extracts are prepared from two different lysogenic strains. An extract containing preheads is prepared from strain BHB2690 by sonication, and an extract containing the D protein and other components necessary for packaging is prepared from strain BHB2688 by freeze-thaw lysis. Packaging is carried out in vitro by mixing the two extracts and then adding bacteriophage λ DNA (Scalenghe et al 1981, B. Hohn, unpubl.).

Sonicated extract from induced BHB2690 cells (prehead donor)

1. Make a subculture of the master stock of *E. coli* BHB2690 Verify its genotype as described on page 2.96.

2. Read the OD_{600} of a 100-ml overnight culture grown at 32°C Place 500 ml of NZM broth, prewarmed to 32°C, in a 2-liter flask, and inoculate with sufficient cells to give an initial OD_{600} of ~0.1 Incubate the culture at 32°C with vigorous agitation (300 cycles/minute in a rotary shaker) until an OD_{600} of ~0.3 is reached (2–3 hours) It is important that the cultures be in the mid-log phase of growth before induction

3. Induce the lysogen by placing the flask in a water bath preheated to 45°C. Swirl the flask continuously for 15 minutes
   Alternatively, induce the culture by immersing the flask in a shaking water bath set at 65°C. As soon as the temperature inside the flask reaches 45°C, transfer the culture to a water bath set at 45°C and incubate for 15 minutes

4. Incubate the induced cells for 2–3 hours at 38–39°C with vigorous agitation (300 cycles/minute in a rotary shaker) Check for successful induction by adding a drop of chloroform to a small sample of culture, it should clear within a few minutes

5. Recover the cells by centrifugation at 4000*g* for 10 minutes at 4°C

6. Drain off as much liquid as possible Remove any remaining medium with a pasteur pipette and Q-tips. Dry the walls of the centrifuge bottle with Kimwipes

7. Add 3.6 ml of freshly prepared sonication buffer. Resuspend the pellet thoroughly, and transfer the resulting homogeneous suspension to a small, clear plastic tube (Falcon 2054 or 2057).

   Sonication buffer

   20 mM Tris · Cl (pH 8.0)
   1 mM EDTA (pH 8.0)
   5 mM β-mercaptoethanol

8. Sonicate in short bursts (10 seconds) at maximum power using a microtip probe. The tube should be immersed in ice water, and the temperature of the sonication buffer should not be allowed to exceed 4°C. Allow the sample to cool for 20–30 seconds between each burst of sonication. Patience is critical! Sonicate until the solution clears and its viscosity decreases

   The amount of sonication is critical, and the clearing and change in viscosity of the solution are not always readily apparent Sonicators vary widely in efficiency and must be tuned for maximum power output Therefore, when preparing these extracts, remove aliquots of the suspension after sonicating for various times Process the aliquots and use them in separate packaging reactions to determine the optimal sonication time

9. Transfer the sonicated sample to a centrifuge tube, and remove debris by centrifugation at 12,000*g* for 10 minutes at 4°C.

10. Add to the supernatant (~3 ml) an equal volume of cold sonication buffer and 1/6 volume of freshly prepared packaging buffer. Dispense 15-μl aliquots into precooled (4°C) 1.5-ml microfuge tubes. Immediately close the caps of the tubes, immerse the tubes briefly in liquid nitrogen, and transfer them to −70°C for long-term storage.

Packaging buffer

6 mM Tris·Cl (pH 8.0)
50 mM spermidine
50 mM putrescine
20 mM $MgCl_2$
30 mM ATP (pH 7.0) (see Appendix B)
30 mM β-mercaptoethanol

Frozen-thawed lysate of induced BHB2688 cells (packaging protein donor)

1. Make a subculture of the master stock of *E. coli* BHB2688. Verify its genotype as described on page 2.96.

2. Read the OD_{600} of a 100-ml overnight culture grown at 32°C. Place 500 ml of NZM broth, prewarmed to 32°C, in each of three 2-liter flasks, and inoculate with sufficient cells to give an initial OD_{600} of ~ 0.1. Incubate the cultures at 32°C with vigorous agitation (300 cycles/minute in a rotary shaker) until an OD_{600} of ~ 0.3 is reached (2–3 hours).

3. Induce the lysogen by placing the flasks in a water bath preheated to 45°C. Swirl the flasks continuously for 15 minutes.

   Alternatively, induce the cultures by immersing the flasks in a shaking water bath set at 65°C. As soon as the temperature inside the flasks reaches 45°C, transfer the cultures to a water bath set at 45°C for 15 minutes.

4. Incubate the induced cells for 2–3 hours at 38–39°C with vigorous agitation (300 cycles/minute in a rotary shaker). Check for successful induction by adding a drop of chloroform to a small sample of the culture; it should clear within a few minutes.

5. Recover the cells by centrifugation at 4000*g* for 10 minutes at 4°C.

6. Drain off as much liquid as possible. Remove any remaining medium with a pasteur pipette and Q-tips. Dry the walls of the centrifuge bottle with Kimwipes.

7. Resuspend the cells in a total of 3 ml of ice-cold sucrose solution (10% sucrose in 50 mM Tris·Cl [pH 8.0]). Dispense 0.5 ml of the suspension into each of six precooled (4°C) microfuge tubes. Add 25 μl of fresh, ice-cold lysozyme solution (2 mg/ml lysozyme in 10 mM Tris·Cl [pH 8.0]) to each tube. Mix gently. Quickly close the caps of the tubes and immerse in liquid nitrogen.

   Lysozyme will not work efficiently if the pH of the solution is less than 8.0.

8. Use forceps to remove the tubes from the liquid nitrogen. Thaw the extracts on ice. Add 25 μl of freshly prepared packaging buffer (see step 10, page 2.101) to each tube and mix.

9. Combine the thawed extracts in a centrifuge tube, and centrifuge at 45,000*g* for 1 hour at 4°C.

10. Dispense 10-μl aliquots of the supernatant into precooled (4°C) microfuge tubes. Immediately close the caps of the tubes and immerse in liquid nitrogen. After all of the aliquots have been frozen, remove the tubes from the liquid nitrogen and immediately transfer them to long-term storage at −70°C.

Note

The sonicated extract and the frozen-thawed lysate can be combined at the time of preparation, if desired Prepare the sonicated extract first, and freeze 15-μl aliquots in open microfuge tubes arranged in a rack in liquid nitrogen Add 10 μl of frozen-thawed lysate directly into each tube Close the caps of the tubes and immerse in liquid nitrogen Store the tubes at −70°C

The major problem in preparing combined extracts is handling the frozen tubes and attempting to pipette 10 μl of frozen-thawed lysate into a tube at −70°C Because extracts combined before or after freezing are able to package bacteriophage λ DNA with similar efficiencies, we recommend storing the sonicated and frozen-thawed extracts separately

PROTOCOL I: PACKAGING IN VITRO USING TWO EXTRACTS

The following protocol is from Scalenghe et al (1981) and B. Hohn (unpubl.).

1. Remove the tubes from storage at −70°C, and allow the packaging extracts to thaw on ice. The frozen-thawed lysate will thaw first. Transfer the frozen-thawed lysate to the still-frozen sonicated extract.

2. Mix gently When the combined extracts are almost totally thawed, add the DNA to be packaged (up to 1 μg dissolved in 5 μl of 10 mM Tris · Cl [pH 8.0], 10 mM $MgCl_2$). Mix with a fine glass stirring rod (e g., a sealed capillary pipette) Try to avoid introducing bubbles. Incubate the tube for 1 hour at room temperature.

3. Add 0.5–1 ml of SM and a drop of chloroform Mix gently. Remove debris by centrifugation at 12,000*g* for 30 seconds at room temperature in a microfuge.

4. Measure the titer of the viable bacteriophage particles by plating on the appropriate indicator strains as described on page 2 61.

   The titer of the bacteriophage stock remains stable for at least 6 months when the packaging reactions are stored in closed tubes at 4°C

Notes

i Each batch of extracts should be tested for packaging efficiency with a standardized preparation of intact bacteriophage λ DNA.

ii These extracts exhibit a high degree of selectivity in the size of the DNA that is packaged (Sternberg et al 1977) Recombinant DNAs that are 90% or 80% of wild-type bacteriophage λ in length are packaged with efficiencies that are 20-fold to 50-fold lower, respectively, than those obtained with unit-length bacteriophage λ

iii. The same packaging extracts may be used for the packaging of both bacteriophage λ and cosmids

PROTOCOL II: PREPARATION OF PACKAGING EXTRACTS FROM ONE LYSOGEN

This system has been described in great detail by Rosenberg (1985, 1987). It uses a single strain of bacteria, SMR10 (λ*cos*2 *ΔB xis*1 *red*3 *gam*am210 *c*I*ts*857 *nin*5 *S*am7/λ), which is a nonsuppressing lysogen of *E. coli* C. Cultures established from single colonies of this strain should be checked for inducibility as described on page 2.96.

1. Grow a 50-ml culture of SMR10 overnight at 34°C. Measure the OD$_{600}$. Place 115 ml of LB broth, prewarmed to 34°C, in each of four 2-liter flasks and inoculate with sufficient cells to give an initial OD$_{600}$ of 0.015. Grow the cells at 34°C with vigorous agitation (300 cycles/minute in a rotary shaker) until the OD$_{600}$ reaches 0.8 (2.5–3 hours).

2. While the cells are growing, prepare the following mixture in a prechilled microfuge tube on ice.

H_2O	255 μl
DMSO	300 μl
0.1 M ATP (pH 7.0) (see Appendix B)	45 μl

Addition of DMSO to water generates heat, so allow the mixture to cool on ice for several minutes before adding the ATP.

3. Dispense 5 μl of the mixture into each of 60 microfuge tubes on ice. Store the tubes on ice.

4. When the cultures have grown to OD$_{600}$ = 0.8, transfer them to two 2-liter flasks prewarmed to 45°C. Shake the cultures vigorously at 45°C for 15 minutes.

5. Transfer the flasks to a shaking incubator and incubate for 90 minutes at 37°C with vigorous agitation.

6. Cool the cultures quickly by swirling the flasks in ice water for 5 minutes. Do not allow the cells to warm up from this point onward.

7. Transfer the cultures to two precooled 250-ml centrifuge bottles, and centrifuge at 4000*g* for 6 minutes at 4°C in a precooled rotor.

8. Drain off as much liquid as possible. Remove any remaining medium with a pasteur pipette and Q-tips. Dry the walls of the centrifuge bottle with Kimwipes. This step is best performed in a cold room.

9. Add 4.5 ml of precooled TSP (see page 2.106) to each of the two bacterial pellets. Using a chilled pasteur pipette, gently resuspend the bacteria. Transfer both suspensions into a single, precooled, 12-ml centrifuge tube.

TSP

0.04 M Tris · Cl (pH 7.9)
0.01 M spermidine
0.01 M putrescine

10. Centrifuge at 4000*g* for 6 minutes at 4°C in a precooled rotor. Carefully remove all the supernatant as described in step 8

11. Add 0.35 ml of precooled TSP to the pellet, and gently resuspend it using a thin glass rod. This step is best carried out in a cold room The suspension should be thick and pasty, but not viscous

12. Use a 50-μl disposable glass micropipette to dispense 20 μl of this suspension into each prechilled microfuge tube containing the DMSO/ATP solution prepared in step 2 Vortex each tube briefly as the extract is added Store the open tubes at 0°C until all of the suspension has been dispensed

13. Freeze the extracts by placing the open tubes in liquid nitrogen

14. Cap the tubes and store in liquid nitrogen. The packaging efficiency of the extract should remain constant for at least 4 months under these conditions Storage at −70°C results in a marked drop in efficiency within 1 week

PROTOCOL II: PACKAGING IN VITRO USING ONE EXTRACT

1. Using forceps, remove the required number of tubes of packaging extract from the liquid nitrogen, and immediately add 10 μl of DNA. Immediately thaw the extracts by placing the open tubes in a water bath set at 28°C for 30 seconds.

   A packaging efficiency of 0 2 $\times 10^8$ to 1 $\times 10^8$ pfu per microgram of bacteriophage λ DNA is routinely obtained This efficiency is independent of the amount of DNA added within the range of 10 pg to 4 μg

2. Mix the contents of the tubes carefully, using a thin glass stirring rod (e.g , a sealed capillary pipette) Try to avoid introducing bubbles. Incubate the tubes for 90 minutes at 28°C.

3. When the packaging reaction is complete, add 0 5 ml of SMC containing 50 μg/ml DNAase I Mix well Add 3 μl of chloroform and mix again. Vortex the tubes until no solid material is visible and each lysate is homogeneous.

   To prepare SMC, mix.

SMC-A	8 ml
SMC-B	2 ml
sterile H_2O	190 ml

   Do not autoclave SMC

   SMC-A

anhydrous Na_2HPO_4	52.5 g
KH_2PO_4	22.5 g
H_2O to 300 ml	

   Sterilize by autoclaving for 15 minutes at 15 lb/sq. in. on liquid cycle.

   SMC-B

NaCl	10 g
NH_4Cl	20 g
1 M $MgCl_2$	20 ml
1 M $CaCl_2$	2 ml
H_2O to 200 ml	

   Sterilize by autoclaving for 15 minutes at 15 lb/sq. in. on liquid cycle.

4. Centrifuge at 12,000g for 2 minutes at room temperature in a microfuge, and collect the supernatant

5. Measure the titer of the viable bacteriophage particles by plating on the appropriate indicator strains as described on page 2 61

   The titer of the bacteriophage stock remains stable for at least 6 months when the packaging reactions are stored in closed tubes at 4°C

Identification and Analysis of Recombinants

IN SITU HYBRIDIZATION OF BACTERIOPHAGE λ PLAQUES

After a collection of recombinant bacteriophages has been prepared, it is necessary to identify and isolate specific desired recombinants from the population. The most commonly used method involves screening of bacteriophage plaques by hybridization with ^{32}P-labeled DNA probes (Benton and Davis 1977) Bacteriophages are plated at an appropriate density, and an imprint of the pattern of plaques is obtained by gently layering a nitrocellulose filter (or nylon membrane) onto the surface of the top agarose Bacteriophage particles and DNA are transferred to the filter by capillary action in an exact replica of the pattern of plaques After denaturation with alkali, the DNA is irreversibly bonded to the filter by baking and is then hybridized to the ^{32}P-labeled probe. Excess probe is washed away, and the filters are then exposed to autoradiographic film Hybridizing plaques, identified by aligning the film with the original agar plate, are picked for further analysis This method is particularly valuable for identifying small numbers of recombinant bacteriophages that carry sequences of interest in complex cDNA or genomic DNA libraries (Sim et al 1979)

To screen a library of mammalian DNA (genome complexity 3×10^9 bp), several hundred thousand recombinant plaques must be examined Table 2 6 gives the maximum number of plaques that can be screened in culture dishes of different sizes In the following example, the volumes given are suitable for screening approximately 50,000 plaques in a 150-mm petri dish

TABLE 2.6 Numbers of Plaques in Culture Dishes of Various Sizes

Size of petri dish (mm)	Total area (cm^2)	Volume of bottom agar (ml)	Volume of indicator bacteria (ml)	Volume of top agarose (ml)	Maximum number of plaques/dish
90	63.9	30	0.1	2 5	15,000
150	176 7	80	0 3	6 5	50,000

Immobilization of Bacteriophage λ Plaques on Nitrocellulose Filters or Nylon Membranes

This method is modified from that of Benton and Davis (1977)

1. Mix aliquots of a packaging reaction or bacteriophage λ stock containing no more than 50,000 bacteriophage particles in a volume of 50 μl or less with 0 3 ml of plating bacteria (see page 2.60). Incubate the infected bacteria for 20 minutes at 37°C

2. Add to each aliquot 6.5 ml of molten (47°C) top agarose (0 7%) and pour onto a 150-mm agar plate The plates must be dry; otherwise, the layer of top agarose will peel off when the filter is removed Usually, 2-day-old plates that have been dried for several additional hours at 37°C with the lids slightly open work well In humid weather, however, incubation for 1 day or more at 41°C may be necessary

   Be sure to use top agarose rather than top agar, since agar peels off even more easily than agarose

3. Incubate the plate containing the infected bacteria at 37°C until the plaques reach a diameter of approximately 1 5 mm and are just beginning to make contact with one another (10–12 hours) The plate should not show confluent lysis

4. Chill the plates at 4°C for at least 1 hour to allow the top agarose to harden

5. Number nitrocellulose filters (Millipore HATF or HAWP, Schleicher and Schuell BA85, or equivalent) or nylon membranes (Schleicher and Schuell, Nytran, or equivalent) with a soft-lead pencil or a ballpoint pen

   If the filters will be hybridized to a number of different probes, it is better to use nylon membranes rather than nitrocellulose filters Nylon membranes are more pliable than nitrocellulose filters and withstand repeated exposure to extremes of temperature far better Nylon membranes are also preferred when hybridization is to be carried out in solvents containing tetramethylammonium chloride (see Chapter 11) However, different brands of nylon membranes vary in their ability to bind DNA, in the ease with which they can be stripped of radiolabeled probes, and in the degree to which they distort during repeated rounds of stripping and rehybridization (Khandjian 1987) It would therefore be sensible to check the properties of nylon membranes obtained from different manufacturers before attempting large-scale screening of bacteriophage λ libraries Follow the manufacturer's recommendations closely, since they have presumably been shown to lead to optimal results The protocol given below works well with all commercial brands of nitrocellulose filters

6. Remove the plates from the cold room or refrigerator, and, at room temperature, place a dry, circular nitrocellulose filter neatly onto the surface of the top agarose so that it comes into direct contact with the plaques Be careful not to trap air bubbles The filter should be handled with gloved hands, finger oils prevent wetting of the filter and affect transfer of DNA. Mark the filter in three or more asymmetric locations

by stabbing through it and into the agar beneath with an 18-gauge needle attached to a syringe containing waterproof black drawing ink.

Once in contact with the top agarose, the filter wets very rapidly and transfer of bacteriophage DNA occurs quickly. Therefore, do not move the filter once contact with the plate is made. The easiest way to place the filter on the plate is to hold it by its edges, bending it slightly so that the middle of the filter makes contact with the center of the plate. Wetting action then pulls the filter onto the plate. Make certain that the keying marks are asymmetrically placed and that both the filter and the plate are marked. There must be enough ink on the plate to be easily visible when a second filter is in place. Large blotches of ink, however, are undesirable.

7. After 30–60 seconds, use blunt-ended forceps (e.g., Millipore forceps) to peel off the first filter and immerse it, DNA side up, in a shallow tray of denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 1–5 minutes. Transfer the filter into neutralizing solution (1.5 M NaCl, 0.5 M Tris·Cl [pH 7.4]) for 5 minutes. Rinse the filter in 2 × SSC, and place it, DNA side up, on paper towels to dry.

If any top agarose peels off the plate and adheres to the filter, remove it by gently agitating the filter in denaturing solution.

G. Struhl (unpubl.) has developed the following procedure to fix bacteriophage λ DNA to nitrocellulose filters that avoids treating filters with alkali and neutralizing solution. This can save time when dealing with large numbers of filters.

a. After removing the filters from the top agarose, place them, DNA side up, on paper towels for 5–10 minutes.
b. When the edges of the filters begin to curl, place them in stacks of ten interleaved with circular Whatman 3MM papers. Place a few 3MM papers on the top and bottom of the stack.
c. Place the stacks on a small platform (e.g., the bottom of a Pyrex dish) in an autoclave. Expose to "streaming steam" for 3 minutes (i.e., 100°C—avoid superheated steam).
d. Transfer the stack of filter papers and nitrocellulose filters to a vacuum oven. Bake for 2 hours at 80°C while drawing a vacuum continuously. Any papers that stick to the nitrocellulose filters can be removed by soaking in 2 × SSC before prehybridizing.

When many filters are to be processed at one time, an alternate procedure is to transfer them from the surfaces of the agarose plates to a large piece of 3MM paper soaked in denaturing solution. Plastic cafeteria trays can be used to hold the 3MM paper. After 2 minutes, quickly transfer the filters to a fresh piece of 3MM paper soaked in neutralizing solution. When transferring the filters, use the edge of the first cafeteria tray to remove as much fluid as possible from the underside of the filters. After 3 minutes, transfer the filters to a third sheet of 3MM paper that has been soaked in neutralizing solution. After 3 minutes, rinse the filters briefly in 2 × SSC and place them on paper towels to dry.

8. Place a second dry filter onto the same plate and mark it with waterproof black drawing ink at the same locations as in step 6. After 1–2 minutes, peel off the filter, denature the DNA, and neutralize as described in step 7.

   Generally, the first filter is left in contact with the plaques for 30–60 seconds and subsequent filters are left in contact about 30–60 seconds longer, or until the filter is completely wet. As many as seven replicas have been prepared from a single plate (Benton and Davis 1977), but the strength of the hybridization signal decreases significantly after the third filter.

9. After all of the filters are dry (usually 30–60 minutes at room temperature), sandwich them between sheets of 3MM paper. Fix the DNA to the filters by baking for 30 minutes to 2 hours at 80°C in a vacuum oven.

   Overbaking can cause the filters to become brittle Filters that have not been completely neutralized turn yellow or brown during baking and chip very easily The background of nonspecific hybridization also increases dramatically

   Baking at 80°C is not necessary to fix DNA to some types of nylon membranes (see manufacturer's instructions)

10. Hybridize the DNA immobilized on the filters to a ^{32}P-labeled probe as described on pages 2.114–2 117

    Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature

Immobilization of Bacteriophage λ Plaques on Nitrocellulose Filters Following In Situ Amplification

Woo et al (1978) and Woo (1979) have described a modification of the Benton and Davis (1977) screening method that involves amplification of the bacteriophages on the nitrocellulose filter prior to hybridization Because more bacteriophage DNA becomes attached to the nitrocellulose filter, the autoradiographic signals from positive clones are enhanced approximately fivefold Amplification therefore leads to an improvement in the ratio of signal to noise and also allows the length of the autoradiographic exposure to be reduced Amplification is particularly valuable when oligonucleotides of high degeneracy are used as probes (see Chapter 11) or when only a small amount of a valuable probe is available Extra caution is required to minimize the background of nonspecific hybridization caused by the florid bacterial growth on the filter.

1. Plate out the bacteriophages that are to be screened as described on page 2 109, steps 1–2. However, use 0 3 ml of plating bacteria per 90-mm plate and 1 ml of plating bacteria per 150-mm plate so that the bacterial lawn grows rapidly. Incubate the plates containing the infected bacteria at 37°C

2. Prepare a set of numbered, sterile nitrocellulose filters (Millipore HATF or HAWP, Schleicher and Schuell BA85, or equivalent) if aqueous solvents are to be used in the hybridization mixture If tetramethylammonium chloride is to be used in the hybridization mixture or washing solution, nylon membranes may be required (see Chapter 11)

   To prepare filters, number dry nitrocellulose filters with a soft-lead pencil or a ballpoint pen, wet them with water, and sandwich them between dry Whatman 3MM papers Wrap the stack of filters in aluminum foil, and sterilize them by autoclaving (15 lb/sq in on liquid cycle)

3. When the plaques are about 0 5 mm in diameter, remove the plates from the incubator and chill at 4°C for at least 1 hour to allow the top agarose to harden

4. Gently place a nitrocellulose filter onto the surface of one of the plates Key the filter to the plate with marks made with waterproof black drawing ink (see step 6, page 2 109) Store the plate in the refrigerator for 5 minutes to allow transfer of the bacteriophages and bacteria to take place

5. Remove the filter from the plate and transfer it to a fresh agar plate. The side that was in contact with the bacteriophage plaques should face upward

6. Repeat steps 4 and 5 with a second and third filter, if desired.

7. Wrap the master plates in Parafilm and store at 4°C in an inverted position until the results of the hybridization reaction are available.

8. Incubate the replica plates at 37°C until a thick bacterial lawn with clearly visible plaques has appeared on the filter (5–12 hours).

   If the bacterial lawn is not dense at this stage, the bacteriophage plaques can streak during subsequent transfer.

   Bacterial growth is more rapid on nitrocellulose filters than on nylon membranes.

   In the original procedure described by Woo et al. (1978), the filters were soaked in a suspension of bacteria and then dried before they were applied to the bacteriophage plaques. This soaking is unnecessary, since sufficient bacteria are transferred from the dense lawn to allow several more rounds of bacteriophage replication.

9. Saturate a piece of Whatman 3MM paper in a baking or petri dish with denaturing solution (0.5 N NaOH, 1.5 M NaCl). Pour off the excess liquid. Remove the filters from the plates and place them, plaque side up, on the saturated 3MM paper for 5 minutes.

10. Transfer each filter to a piece of 3MM paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris·Cl [pH 7.4]). After 5 minutes, transfer the filters to a fresh sheet of 3MM paper also saturated with neutralizing solution. After 5 minutes, transfer the filters, plaque side up, to paper towels to dry.

    When transferring the filters, use the edge of the dish to scrape as much fluid as possible from the underside of the filters.

11. After all the filters are dry (usually 30–60 minutes at room temperature), sandwich them between sheets of 3MM paper. Fix the DNA to the filters by baking for 30 minutes to 2 hours at 80°C in a vacuum oven.

    Overbaking can cause the filters to become brittle. Filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

    Baking at 80°C is not necessary to fix DNA to some types of nylon membranes (see manufacturer's instructions).

12. Hybridize the DNA immobilized on the filters to a ^{32}P-labeled probe as described on pages 2.114–2.117.

    Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

    To reduce background, wash the filters before hybridization for 30–60 minutes in 5 × SSC, 0.5% SDS at 65°C. Then gently scrape the bacterial debris from the surface using Kimwipes soaked in the same buffer. Neither the intensity of the hybridization signal nor its sharpness is affected by this procedure. After removing the bacterial debris, rinse the filters in 5 × SSC, 0.5% SDS, and then prehybridize and hybridize the filters as described on pages 2.114–2.117.

Hybridization to Nitrocellulose Filters Containing Replicas of Bacteriophage λ Plaques

For a general discussion of the factors that affect the rate and specificity of hybridization of radioactive probes to nucleic acids immobilized on solid supports, see Chapter 9, pages 9.47–9.51.

The following protocol is designed for 30 circular filters, 82 mm in diameter. Appropriate adjustments should be made to the volumes when carrying out hybridization reactions with different numbers or sizes of filters

1. Float the baked filters on the surface of a tray of 2 × SSC until they have become thoroughly wetted from beneath Submerge the filters for 5 minutes

2. *Optional.* Transfer the filters to a circular glass crystallizing dish containing 100 ml of prewashing solution Stack the filters on top of one another in the solution, and cover the dish with Saran Wrap In this and all subsequent steps, the filters should be slowly agitated on a rotating platform in a water bath or incubator at the appropriate temperature to prevent them from sticking to one another

   Important: Do not allow the filters to dry at any stage during the prewashing, prehybridization, or hybridization steps

   Prewashing solution

   5× SSC
   0.5% SDS
   1 mM EDTA (pH 8.0)

   Incubate the filters for 1–2 hours at 42°C Then, gently scrape the bacterial debris from the surfaces of the filters using Kimwipes soaked in the prewashing solution. This reduces background hybridization without affecting the intensity or sharpness of positive signals

3. Transfer the filters to a glass crystallizing dish containing 60 ml of prehybridization solution Incubate for 1–2 hours at the appropriate temperature (68°C when hybridization is to be carried out in aqueous solution, 42°C when hybridization is to be carried out in 50% formamide)

   Some workers prefer to incubate the filters in sealed plastic bags (Sears Seal-A-Meal or equivalent) (see, e g , Chapter 9, page 9 53) This method avoids problems of evaporation and, because the sealed bags can be submerged in a water bath, ensures that the temperatures during hybridization and washing are correct The bags must be opened and resealed when changing buffers To avoid radioactive contamination of the water bath, the resealed bag containing radioactivity should be sealed inside a second, noncontaminated bag

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases It is important that these bubbles be removed by occasionally agitating the fluid in the bag, otherwise, the components of the prehybridization solution will not be able to coat the filter evenly This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag

   The filters should be completely covered by the prehybridization solution. During prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become blocked by proteins in the BLOTTO

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A) at a concentration of 1 μg/ml should be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference Both versions of these solutions give excellent results and neither has clear-cut advantages over the other However, hybridization in 50% formamide at 42°C is less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution Offsetting this advantage is the two- to threefold slower rate of hybridization in solutions containing formamide

To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below T_m (see Chapter 9, pages 9 50–9 51) Both solutions work equally well when hybridization is carried out in aqueous solvents However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power

Prehybridization solution

Either
50% formamide
6× SSC (or 6× SSPE)
0.05× BLOTTO
or
6× SSC (or 6× SSPE)
0.05× BLOTTO

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

1 × BLOTTO: Bovine Lacto Transfer Technique Optimizer (Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C. 1 × BLOTTO is as effective a blocking agent as Denhardt's reagent, but much less expensive. It should not be used in combination with high concentrations of SDS, which will cause milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent when radiolabeled RNA is used as a hybridization probe, because of the possibility that the dried milk may contain significant amounts of RNAase activity.

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

4. Denature ^{32}P-labeled double-stranded DNA probe by heating for 5 minutes to 100°C. Chill the probe rapidly in ice water. Single-stranded probe need not be denatured. Add the probe to the prehybridization solution covering the filters Incubate at the appropriate temperature until 1–3 × $C_0t_{1/2}$ is achieved (see Chapter 9, page 9.48). During the hybridization, the containers holding the filters should be tightly closed to prevent loss of fluid by evaporation.

   Alternatively, the probe may be denatured by adding 0 1 volume of 3 N NaOH After 5 minutes at room temperature, transfer the probe to ice water and add 0 05 volume of 1 M Tris Cl (pH 7 2) and 0 1 volume of 3 N HCl Store the probe in ice water until it is needed.

   Between 2×10^5 and 1×10^6 cpm of ^{32}P-labeled probe (sp act $\geq 5 \times 10^7$ cpm/μg) should be used per milliliter of prehybridization solution Using more probe will cause the background of nonspecific hybridization to increase, using less will reduce the rate of hybridization

5. When the hybridization is completed, remove the hybridization solution and immediately immerse the filters in a large volume (300–500 ml) of a solution of 2 × SSC and 0.1% SDS at room temperature Agitate the filters gently, and turn them over at least once during washing After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently Repeat the washing procedure twice more At no stage during the washing procedure should the filters be allowed to dry

   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment However, hybridization mixtures containing complementary strands of DNA should be discarded, since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization

6. Wash the filters twice for 1–1 5 hours in 300–500 ml of a solution of 1 × SSC and 0 1% SDS at 68°C At this point, the background is usually low enough to put the filters on film If the background is still high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of a solution of 0 2 × SSC and 0.1% SDS at 68°C

7. Dry the filters in the air on paper towels at room temperature Arrange the filters (numbered side up) on a sheet of Saran Wrap Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap These markers serve to align the autoradiograph with the filters Cover the labels with Scotch Tape This prevents contamination of the film holder or intensifying screen with radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink We find it convenient to make the ink in three grades. very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor) Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels Attach radioactive-warning tape to the pen, and store it in an appropriate place

8. Cover the filters with a second sheet of Saran Wrap Expose the filters to X-ray film (Kodak XAR or equivalent) for 12–16 hours at −70°C with an intensifying screen (see Appendix E).

9. Develop the film and align it with the filters using the marks left by the radioactive ink Use a nonradioactive fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters. Tape a piece of clear Mylar or other firm transparent sheet to the film Mark on the clear sheet the positions of positive hybridization signals. Also mark (in a different color) the positions of the asymmetrically located dots Remove the clear sheet from the film. Identify the positive plaques by aligning the dots on the clear sheet with those on the agar plate

   Some batches of nitrocellulose filters swell and distort during hybridization and subsequent drying, so that it becomes difficult to align the two sets of dots This problem can be alleviated to some extent by autoclaving the dry filters between pieces of damp Whatman 3MM paper before use (10 lb/sq in for 10 minutes on liquid cycle) Nylon membranes do not suffer from this problem

10. Each positive plaque should be picked as described on page 2 63 and placed in 1 ml of SM containing a drop of chloroform Often, the alignment of the filters with the plate does not permit identification of an individual hybridizing plaque In this case, an agar plug containing several plaques should be picked. An aliquot (usually 50 μl of a 10^{-2} dilution) of the bacteriophages that elute from the agar plug is replated so as to obtain approximately 500 plaques on a 90-mm plate. These plaques are then screened a second time by hybridization A single, well-isolated positive plaque should be picked from the secondary screen and used to make a plate stock (see page 2 65 or 2.66)

RAPID ANALYSIS OF BACTERIOPHAGE λ ISOLATES

After specific clones have been identified by hybridization, it is important to characterize them by restriction endonuclease digestion and gel electrophoresis before proceeding further. Two methods are presented below for the rapid preparation of small amounts of bacteriophage λ DNA that are pure enough to be analyzed by restriction endonuclease digestion: the plate lysate method (E F. Fritsch, unpubl.) and the liquid culture method (Leder et al 1977) Both methods work equally well, although there are occasional problems with the yield and purity of the bacteriophage DNA If trouble should arise, make up new batches of reagents and use a fresh bacterial culture that has been grown from a single colony

Plate Lysate Method

1. Using a pasteur pipette, pick a single, well-isolated bacteriophage plaque (see page 2.63) and place in 1 ml of SM containing a drop of chloroform in a small, sterile tube Store the suspension for 4–6 hours at 4°C to allow the bacteriophage particles to diffuse out of the top agarose

2. In a small, sterile culture tube, mix 50–100 μl of the bacteriophage suspension ($\sim 10^5$ pfu) with 100 μl of plating bacteria (see page 2 60) Incubate for 20 minutes at 37°C Add 2 5 ml of molten (47°C) top agarose (0 7%), and spread the bacterial suspension on the surface of a freshly poured 90-mm plate containing 30 ml of NZCYM plus 1 5% agarose

   Freshly poured plates give much better yields of bacteriophage than older, dry plates Do not use agar to make the bottom medium, since most batches contain potent inhibitors of restriction enzymes

3. Invert the plate and incubate at 37°C until the plaques cover almost the entire surface of the plate (9–14 hours)

4. Add 3 ml of λ diluent directly onto the surface of the top agarose, and allow the bacteriophage particles to elute during 1–2 hours of storage at room temperature with constant, gentle shaking

   λ diluent is used rather than SM because it lacks gelatin, which is sometimes difficult to remove by extraction with phenol

   λ Diluent

   10 mM Tris · Cl (pH 7.5)
   10 mM $MgSO_4$

5. Transfer the λ diluent to a centrifuge tube, and remove the bacterial debris by centrifugation at 4000*g* for 10 minutes at 4°C

6. Transfer the supernatant to a centrifuge tube, and add 1 μl each of RNAase A (1 mg/ml) and DNAase I (1 mg/ml). Incubate the mixture for 15 minutes at 37°C.

7. Add an equal volume of a solution containing 20% w/v polyethylene glycol (PEG 8000) and 2 M NaCl in λ diluent. Mix by gentle vortexing, and incubate for 1 hour at 0°C (i e , in ice water).

8. Recover the precipitated bacteriophage particles by centrifugation at 10,000*g* for 10 minutes at 4°C

9. Remove the supernatant by gentle aspiration Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.

10. Add 0 5 ml of TE (pH 8 0), and resuspend the bacteriophage particles by vortexing If any debris is visible, remove it by brief centrifugation and discard

11. Add 5 μl of 10% SDS, and incubate for 5 minutes at 68°C

12. Add 10 μl of 5 M NaCl Purify the bacteriophage DNA by extracting once with phenol.chloroform and once with chloroform alone Transfer the aqueous phase to a fresh microfuge tube between the extractions.

13. Add an equal volume of isopropanol to the aqueous phase, and mix. Store at −70°C for 15 minutes Recover the DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge

14. Wash the pellet with 70% ethanol, and recentrifuge briefly Remove the supernatant by gentle aspiration, and allow the pellet to dry at room temperature for a few minutes

15. Dissolve the DNA in 50 μl of TE (pH 8 0)

16. Place 10 μl of the solution in a fresh microfuge tube. Add 1 2 μl of the appropriate restriction enzyme buffer and 1–2 units of the desired restriction enzyme Incubate for 1–2 hours at the appropriate temperature. Store the remainder of the preparation at −20°C

17. Analyze the DNA fragments in the restriction digest by gel electrophoresis

Notes

i Two problems commonly arise with this method First, the yield of bacteriophage λ DNA may be low; second, the DNA may be resistant to cleavage by restriction enzymes The yield of DNA depends partly on the strain of bacteriophage λ and partly on the conditions of growth We have found that the method works with all strains of bacteriophage λ in common use as vectors, except those that give rise to *red*$^-$ *gam*$^-$ recombinants, which simply do not grow to sufficiently high titer For slower-growing bacteriophages, it may sometimes be necessary to adjust the ratio of bacteria to bacteriophages in the inoculum.

If the bacteriophage DNA cannot be cleaved with restriction enzymes, we suggest adding DNAase-free pancreatic RNAase (20 μg/ml) (see Appendix B) to the restriction enzyme buffer or increasing the volume of the restriction digest to 100 μl as follows· To 10 μl of the DNA preparation, add 80 μl of TE (pH 8.0), 10 μl of 10 × restriction enzyme buffer, and 1 μl of restriction enzyme After incubation for 12 hours at the appropriate temperature, add 10 μl of 3 M sodium acetate (pH 5 2) and 220 μl of ethanol. After storage for 30 minutes, recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge. Remove the supernatant by gentle aspiration, and allow the pellet to dry at room temperature Dissolve the pellet in a small volume of TE (pH 8 0), and analyze the fragments by gel electrophoresis

If problems persist, a number of other, more lengthy procedures are available (e.g., purification of bacteriophage λ particles on columns of DEAE-cellulose [Helms et al 1985]).

ii The infectivity of DNA prepared in this way and packaged in vitro is identical to that of more highly purified bacteriophage λ DNA

uid Culture Method

In general, bacteriophage λ does not grow to as high a titer in liquid cultures as in plate lysates. This method should therefore be used only with extremely vigorous strains of the bacteriophage.

1. Using a pasteur pipette, pick a single, well-isolated bacteriophage plaque (see page 2.63) and place in 1 ml of SM containing a drop of chloroform in a small, sterile tube. Store the suspension for 4–6 hours at 4°C to allow the bacteriophage particles to diffuse out of the top agarose.

2. In a 25-ml tube, mix 0.5 ml of the bacteriophage suspension ($\sim 3 \times 10^6$ bacteriophages) with 1.6×10^8 bacterial cells in stationary phase. Incubate for 15 minutes at 37°C. Both the absolute amounts of bacteriophages and bacterial cells and the ratio between them are important. It is best to use bacteriophage stocks or plaque eluates of known titer.

3. Add 4 ml of NZCYM medium, and incubate for approximately 9 hours at 37°C with agitation. The culture should clear, but very little debris should be evident.

4. Add 0.1 ml of chloroform to the culture, and continue incubation for a further 15 minutes at 37°C with agitation. Transfer the lysate to a 5-ml polypropylene centrifuge tube. Centrifuge at 8000*g* for 10 minutes at 4°C.

5. Continue with the plate lysate method at step 6 (see page 2.118).

Notes

i. If lysis does not occur, or is incomplete, add an equal volume of prewarmed NZCYM medium to the cultures and continue incubation for a further 2–3 hours at 37°C with vigorous agitation (300 cycles/minute in a rotary shaker).

ii. Although most strains of bacteriophage λ grow well in liquid culture and routinely yield lysates containing approximately 5×10^9 pfu/ml, there are some strains that replicate poorly. The problem can often be solved by increasing the ratio of bacteriophage to bacteria in the infection or by growing the infected culture at 39°C—the optimum temperature for growth of the bacteriophage. In addition, some workers prefer to carry out the adsorption step at 0°C in the presence of 5 mM $CaCl_2$ (Patterson and Dean 1987). This allows a more uniform infection and synchronous growth of the bacteriophage.

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3
Cosmid Vectors

Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA As discussed in Chapter 9, the number of recombinant clones required to form a complete library of genomic DNA is fixed not only by the size of the genome, but also by the capacity of the vector. The larger the fragment of DNA that can be propagated in the vector, the smaller the number of recombinants in the library The availability of vectors of large capacity was expected to reduce significantly the labor involved in the construction, propagation, and screening of genomic DNA libraries of organisms with large genomes (Collins and Hohn 1978) Unfortunately, because of technical problems (see below), these hopes have been realized only partially Nevertheless, the large capacity of cosmid vectors has proved useful in two particular circumstances:

1 *To clone and propagate an entire eukaryotic gene within a single recombinant.* Many genes of higher eukaryotes contain large and numerous introns and are thus too big to be cloned and propagated as a single segment of DNA in bacteriophage λ or plasmid vectors For example, the genes coding for chicken pro α2 collagen (Ohkubo et al. 1980, Vogeli et al 1980; Wozney et al. 1981), murine dihydrofolate reductase (DHFR) (Nunberg et al 1980), and human steroid sulfatase (Yen et al 1987) are all at least 40 kb in length, much larger than the maximum size of foreign DNA (~24 kb) that can be propagated in bacteriophage λ vectors Although plasmid vectors can, in theory, carry larger inserts, the resulting recombinants transform *Escherichia coli* very inefficiently Plasmids carrying large fragments of DNA are therefore likely to be underrepresented in genomic DNA libraries

2 *To clone and analyze a region of eukaryotic DNA encompassing a family of genes* Even a relatively simple family of genes (e g , the mammalian globin genes) is spread over at least 70 kb of genomic DNA (Fritsch et al 1980), and more complex loci may occupy several hundred kilobases (Steinmetz et al 1982) Such large regions of the genome are usually cloned in a series of overlapping recombinants isolated by chromosome "walking" In this technique, a segment of nonrepetitive DNA isolated from one end of a recombinant is used as a probe to identify recombinant clones containing the adjacent sequence This process is repeated until the entire region of interest has been recovered

   Because chromosome walking is a slow process, with each step taking perhaps a month or more, the use of cosmids rather than bacteriophage λ can be a considerable advantage Since the maximum size of foreign DNA that can be propagated in cosmids is approximately 45 kb, the length of a step in a cosmid library is potentially almost double the length of a step in a bacteriophage λ library

The feasibility of cloning large fragments of DNA in cosmids was first established by Royal et al (1979), who isolated two cosmid clones from a cosmid library of chicken DNA. The two clones contained two overlapping fragments that together spanned more than 46 kb of DNA and contained the ovalbumin gene and two ovalbumin-like genes. Since then, cosmid cloning has been used for the construction of gene libraries and the isolation of genes from many species, including *Drosophila* (Meyerowitz et al. 1980; Ish-Horowicz and Burke 1981), mouse (Cattaneo et al. 1981; Poustka et al. 1984; Steinmetz et al 1986), and man (Grosveld et al. 1981, 1982; Lindenmaier et al 1985)

The chief technical problems that originally beset the construction and propagation of genomic DNA libraries in cosmid vectors were:

- Concatenation of the vector, which results in cosmids lacking inserted eukaryotic DNA
- Insertion of more than one segment of foreign DNA into the same recombinant cosmid.
- Instability of foreign DNA sequences Recombination between repeated elements can result in loss or rearrangement of cloned segments of eukaryotic DNA.
- Wide variation in the yield of different recombinant cosmids.
- Differential growth of clones carrying recombinant cosmids, which leads to over- or underrepresentation of genomic DNA sequences in amplified cosmid libraries

Although most of these difficulties have been solved, genomic DNA cloning in cosmids remains a more difficult proposition than cloning in bacteriophage λ, in large part because of the logistical problems involved in handling a large number of recombinant bacterial colonies and screening them by hybridization. Cloning in cosmids is therefore used primarily in circumstances where the twofold greater capacity of cosmids has a significant advantage. Bacteriophage λ continues to be the vector of choice for the routine construction of genomic DNA libraries

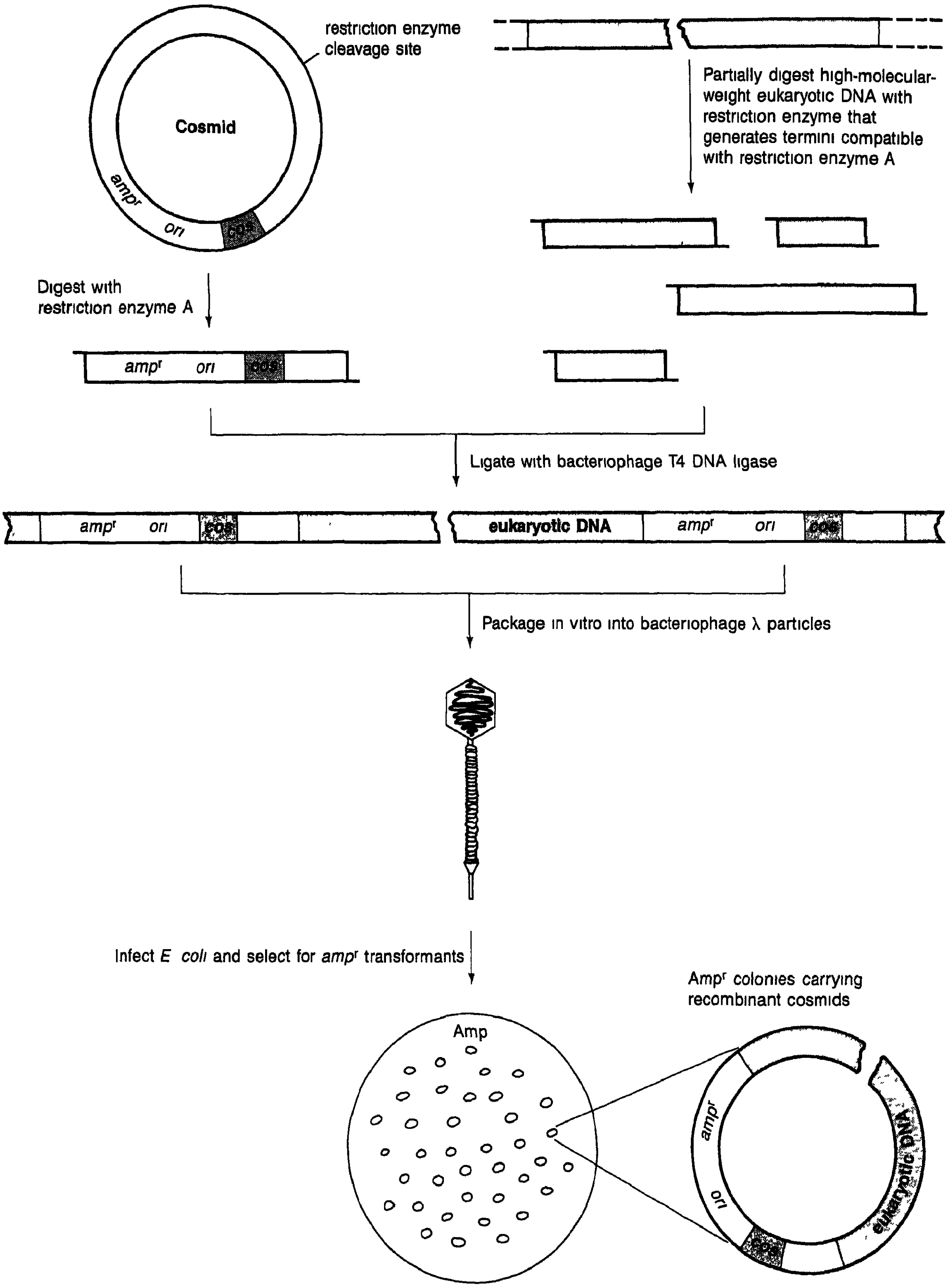

restriction enzyme cleavage site
Cosmid
amp^r
ori
cos
Partially digest high-molecular-weight eukaryotic DNA with restriction enzyme that generates termini compatible with restriction enzyme A
Digest with restriction enzyme A
amp^r
ori
cos
Ligate with bacteriophage T4 DNA ligase
amp^r
ori
cos
eukaryotic DNA
amp^r
ori
cos
Package in vitro into bacteriophage λ particles
Infect E coli and select for amp^r transformants
Amp^r colonies carrying recombinant cosmids
Amp
amp^r
ori
cos
eukaryotic DNA

FIGURE 3.1

The figure shows in diagrammatic form the steps involved in cloning in cosmid vectors Each step is discussed in more detail in the text .

3.4 *Cosmid Vectors*

Cloning in Cosmid Vectors

In their simplest form, cosmid vectors are modified plasmids that carry a copy of the DNA sequences (*cos* sequences) required for packaging DNA into bacteriophage λ particles. Because cosmids carry an origin of replication (usually the ColE1 origin) and a drug resistance marker (usually *amp*^{r}), cosmid vectors can be introduced into *E coli* by standard transformation procedures (see Chapter 1) and propagated as plasmids

To clone in cosmids, segments of foreign DNA approximately 35–45 kb in length are isolated and ligated to linearized vector DNA in vitro under conditions that favor the formation of the type of structure shown in Figure 3.1. Here, the segment of foreign DNA is flanked by cosmid molecules and the two *cos* sites are arranged in the same orientation. Contained between these sites is an entire complement of plasmid genes and the segment of foreign DNA. When concatemers of this type are used as substrates in an in vitro packaging reaction, the *cos* sites are cleaved by the *ter* function of the bacteriophage λ gene *A* protein and the DNA between the two cosmids is packaged into mature bacteriophage λ particles. During infection of *E. coli* by such bacteriophage particles, the linear recombinant DNA is injected into the cell, where it circularizes (via the cohesive ends of the *cos* sites) Because the resulting circular molecule contains a complete copy of the cosmid vector, it replicates as a plasmid and confers drug resistance upon its bacterial host. Bacteria carrying recombinant cosmids can therefore be selected using media containing the appropriate antibiotic.

The packaging process can also be used to transduce a recombinant cosmid from one bacterial host to another Either a bacterial culture carrying a recombinant plasmid is infected with a replication-competent strain of bacteriophage λ or the growth of a resident prophage is induced During the subsequent round of bacteriophage growth, the *cos* sites of the recombinant cosmids serve as substrates for packaging components and the cosmid DNAs become packaged into bacteriophage particles These transducing particles are released from the cell and can be stored indefinitely or used immediately to infect another bacterial strain Genomic DNA libraries constructed in cosmids can therefore be preserved as stocks of bacteriophage λ particles

The cloning capacity of cosmids is a function of (1) the size of the DNA that can be packaged into the head of a λ bacteriophage and (2) the size of the vector itself. Because bacteriophage λ heads will accommodate as much as 52 kb of DNA (Williams and Blattner 1980) and most of the commonly used cosmid vectors are approximately 5 kb in size (see below), recombinant cosmids that contain up to 47 kb of foreign DNA can be constructed and propagated At the other extreme, because the smallest piece of DNA that can be packaged into bacteriophage λ particles is approximately 38 kb (Williams and Blattner 1980), the minimum size of DNA that can be cloned into a conventional cosmid vector (5 kb) is approximately 33 kb. However, a family of modified cosmid vectors (termed "charomids") has been described (Saito and Stark 1986) (see page 3.25) Charomid vectors carry spacer sequences consisting of head-to-tail repeats of a 2-kb fragment derived from pBR322. Different members of this family carry spacers with different numbers of repeating units. Each family member will therefore accommodate an insert whose size falls within the specific limits required to bring the recombinant molecule within the optimal range for packaging into bacteriophage λ particles In this way, charomid vectors can be used to clone fragments of DNA ranging in size from 2 kb to 45 kb When these vectors are propagated in *recA*$^-$ bacteria (e.g., *E coli* strain ED8767), they undergo very little rearrangement, and it is therefore possible to prepare a charomid vector whose capacity is suitable for the task at hand Once an appropriate charomid has been selected, it is used for cloning in exactly the same way as a conventional cosmid.

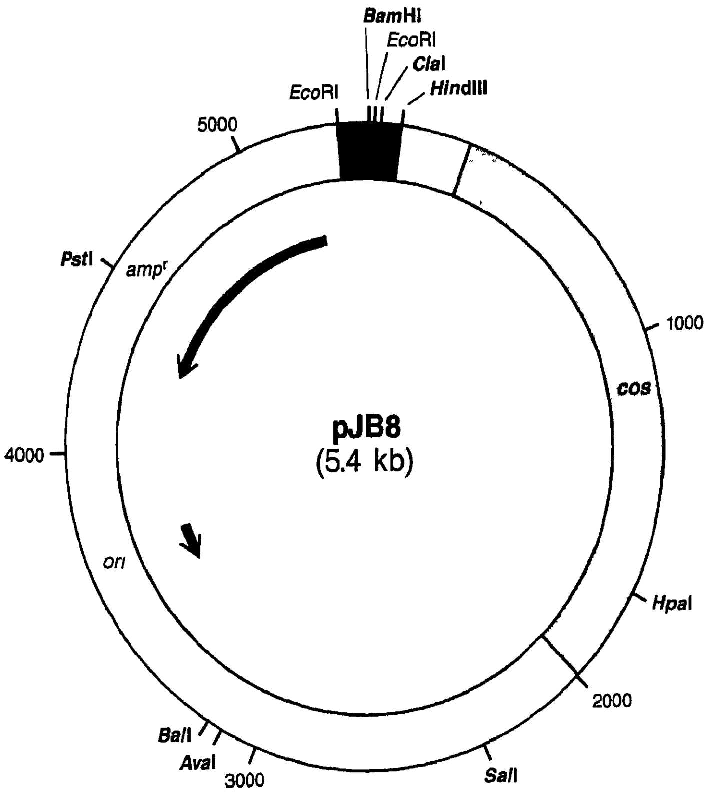
Finally, in addition to their capacity to propagate large pieces of foreign DNA, cosmids have another potentially useful property—the ability to recombine with plasmids that carry homologous sequences Once established in a bacterium, a cosmid can recombine with such a plasmid to form a cointegrant (Poustka et al. 1984; Lindenmaier et al 1985) If the plasmid and cosmid carry independently selectable drug resistance markers and also compatible origins of replication, cointegrants containing both markers can be easily selected following transduction to another bacterium. So far, this method has been used only to a limited extent (Poustka et al 1984, Lindenmaier et al 1985), and there are potential problems that have not been fully explored For example, the recombination event, by necessity, is carried out in a *recA*$^+$ strain of bacteria, thereby creating the opportunity for unwanted rearrangements to occur within the segments of DNA cloned in the cosmid and plasmid vectors However, if such rearrangements can be held within manageable limits, this method may speed up the rate of chromosome walking or, in certain specific cases, may allow the rapid isolation of a single genetic locus from many different individuals.

Cosmid Vectors

In addition to the basic features discussed above that are common to all cosmid vectors, a variety of additional modifications have been made either to alleviate problems of the kind listed on pages 3.54–3 55 or to confer special advantages on individual vectors These modifications include·

- The incorporation of two *cos* sites into a single cosmid vector (Bates and Swift 1983; Poustka et al 1984) As discussed later, the presence of these two sites greatly simplifies directional cloning in cosmid vectors (see Figure 3.3)
- The construction of cosmid vectors that lack homology with commonly used plasmid vectors (Poustka et al 1984, Lindenmaier et al 1985). These vectors are therefore unlikely to recombine with one another when they are carried within the same recombination-competent cell However, if both vectors contain homologous foreign DNA sequences, they recombine in recombination-competent cells to form cointegrants that can be rescued by transformation. This method can be used as a genetic screen to isolate sequences of interest from cosmid libraries
- The presence of promoters for bacteriophage-encoded DNA-dependent RNA polymerases that enable RNA probes specific to either end of the cloned insert to be generated in vitro (Cross and Little 1986; Gibson et al 1987a,b; Wahl et al 1987)
- The incorporation of selectable markers to simplify the identification of eukaryotic cells that have been transfected with recombinant cosmids (see, e g , Bates and Swift 1983, Brady et al 1984; Kioussis et al 1987)
- The incorporation of eukaryotic origins of replication that allow transfected cosmids to replicate autonomously in mammalian cells (Kioussis et al. 1987)
- The addition of a range of prokaryotic markers to facilitate rescue in bacteria of cosmid sequences transfected into mammalian cells (Lund et al 1982, Brady et al 1984)
- The use of bacteriophage origins of replication rather than plasmid origins of replication (Little and Cross 1985, Cross and Little 1986) There is some evidence that bacteriophage origins may alleviate the problems of differential growth displayed by recombinant cosmids carrying ColE1 origins.

No single cosmid vector contains all of these improvements, and it is therefore necessary to choose the particular vector that is most suitable for the task at hand In the following pages, cosmid vectors in common use are described in detail.

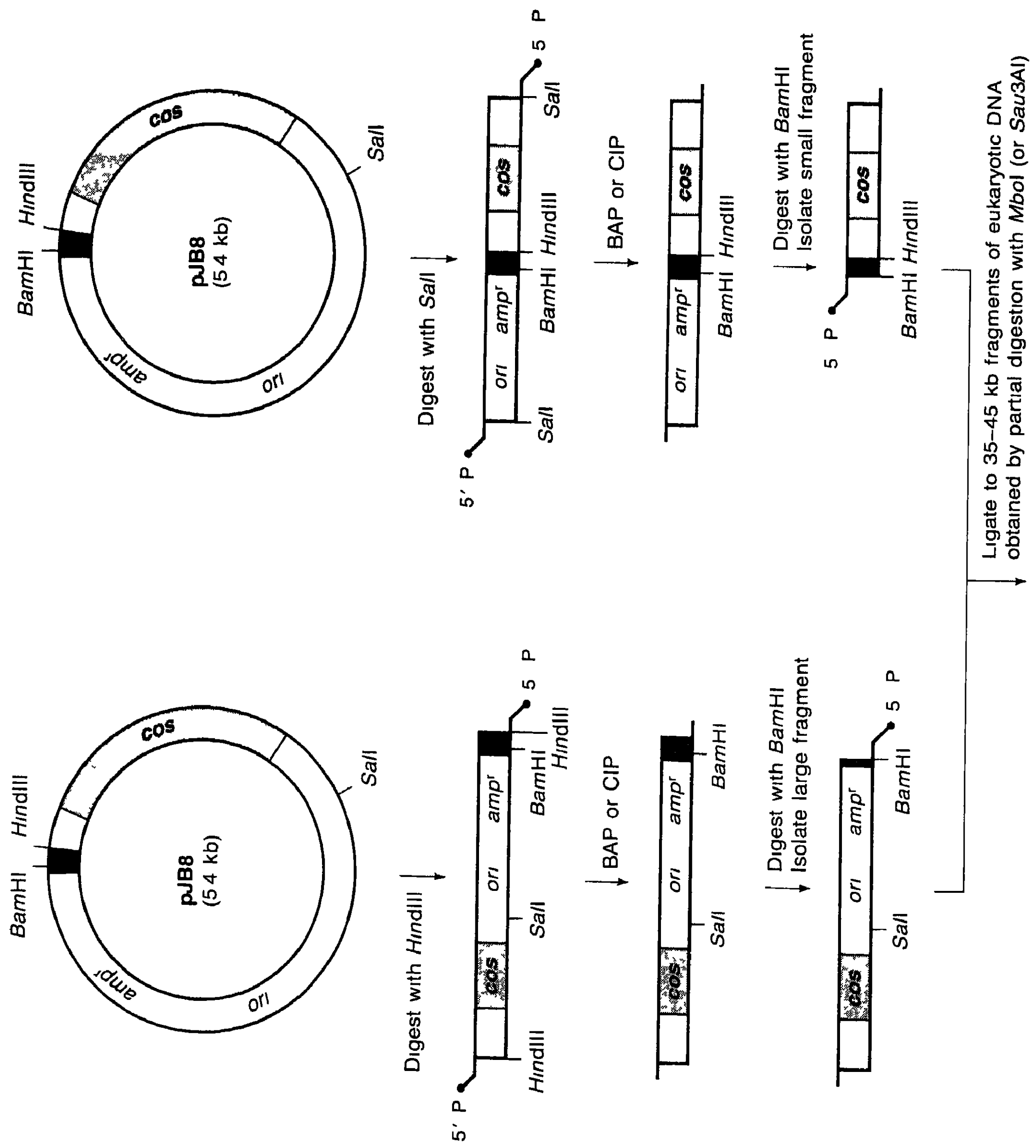

BamHI
EcoRI
ClaI
HindIII
EcoRI
5000
PstI
amp^r
1000
cos
pJB8
(5.4 kb)
4000
ori
HpaI
2000
BalI
AvaI
3000
SalI

COSMID VECTORS FOR PROPAGATION OF EUKARYOTIC DNA IN BACTERIA

pJB8

pJB8 (Ish-Horowicz and Burke 1981) contains an ampicillin resistance (amp^r) gene, a ColE1 origin of replication, a single *cos* site (derived from Charon 4A), and four restriction sites (*Bam*HI, *Eco*RI, *Cla*I, and *Hin*dIII) that can be used for cloning. The vector is approximately 5.4 kb in length and should therefore accept segments of foreign DNA whose lengths lie between 33 kb and 46 5 kb. Concatenation of the vector during ligation to foreign DNA can be suppressed either by treating the linearized vector with phosphatase (see page 3.28) or by using a modified form of directional cloning (see Figure 3 2) However, because this scheme involves a large number of steps, pJB8 is no longer the vector of choice for directional cloning in cosmids. Instead, we recommend vectors that carry two *cos* sites (e.g , c2RB, see page 3.13) Concatemers of pJB8 DNA and foreign DNA are packaged in vitro into bacteriophage λ particles that are used to infect $recA^-$ strains of *E coli*

The two *Eco*RI sites flanking the single *Bam*HI site of pJB8 are particularly useful when "walking" from one recombinant cosmid to another After a recombinant cosmid is isolated from a library, specific subclones representing the extreme ends of the cloned region can easily be derived by digestion of the cosmid DNA with a restriction enzyme that cleaves several times within the foreign · DNA while leaving the vector intact. Following digestion, the remains of the recombinant cosmid are recircularized, generating a small plasmid that contains both ends of the original segment of foreign DNA This plasmid can be reintroduced into bacterial cells by conventional transformation procedures Digestion of the plasmid DNA with *Eco*RI and the second restriction enzyme yields two small DNA fragments that can be used as probes to rescreen the original cosmid library for overlapping clones This approach eliminates the need to map restriction sites or to subclone every part of the segment of foreign DNA in order to identify useful terminal fragments.


BamHI
HindIII
cos
SalI
pJB8
(5.4 kb)
amp^r
ori
Digest with SalI
5′ P
SalI
ori
amp^r
BamHI
HindIII
cos
SalI
5′ P
BAP or CIP
Digest with BamHI
Isolate small fragment
5′ P
BamHI HindIII
cos
Ligate to 35–45 kb fragments of eukaryotic DNA
obtained by partial digestion with MboI (or Sau3AI)
BamHI
HindIII
cos
SalI
pJB8
(5.4 kb)
amp^r
ori
Digest with HindIII
5′ P
HindIII
cos
SalI
ori
amp^r
BamHI
HindIII
5′ P
BAP or CIP
Digest with BamHI
Isolate large fragment
cos
SalI
ori
amp^r
BamHI
5′ P

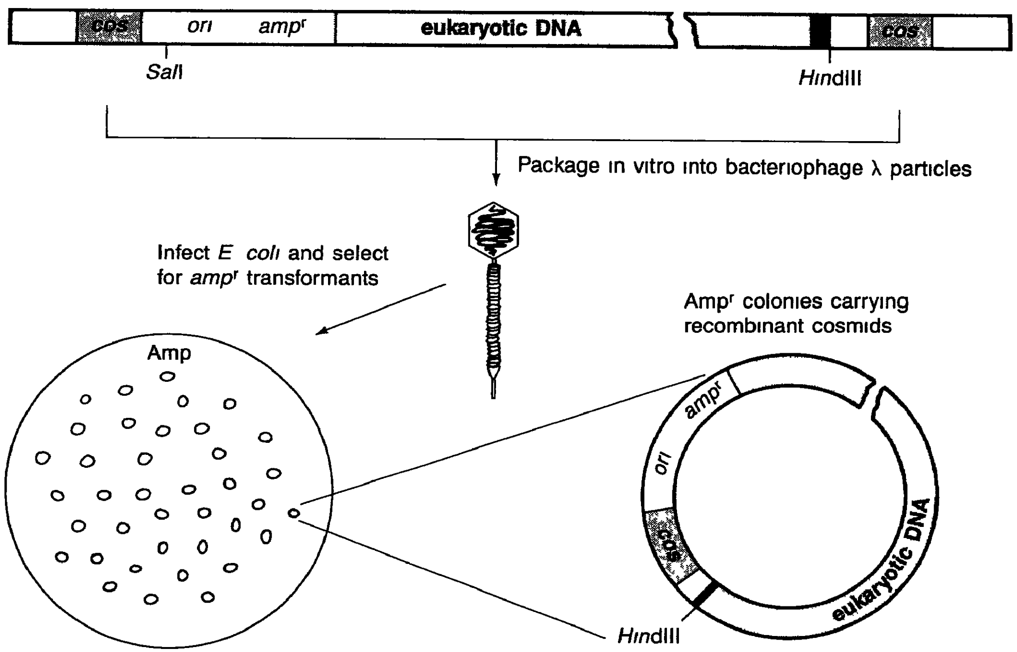
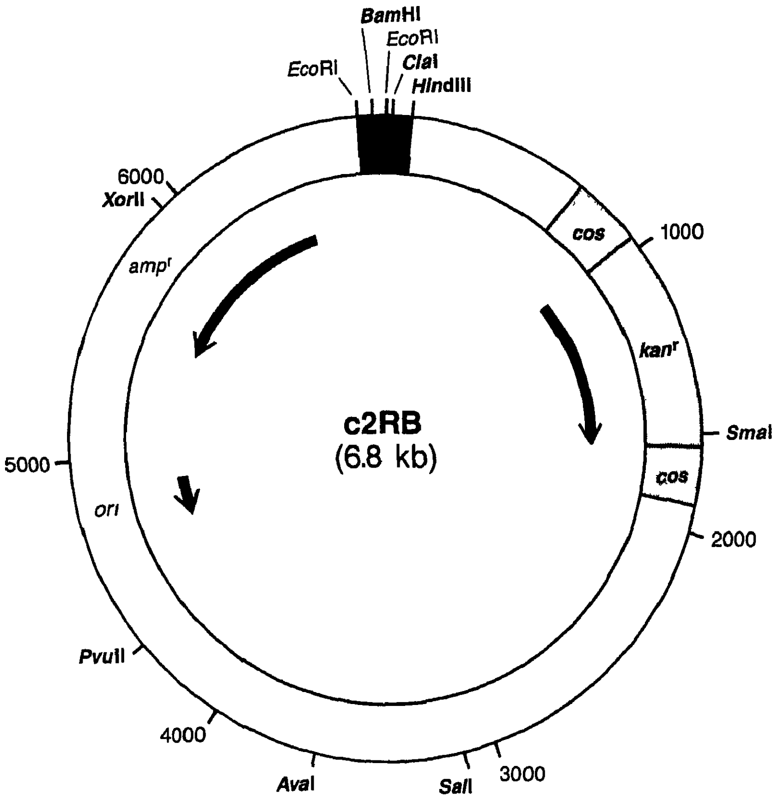

cos
ori
ampr
eukaryotic DNA
cos
SalI
HindIII
Package in vitro into bacteriophage λ particles
Infect E coli and select for ampr transformants
Amp
Ampr colonies carrying recombinant cosmids
ampr
ori
cos
eukaryotic DNA
HindIII

FIGURE 3.2

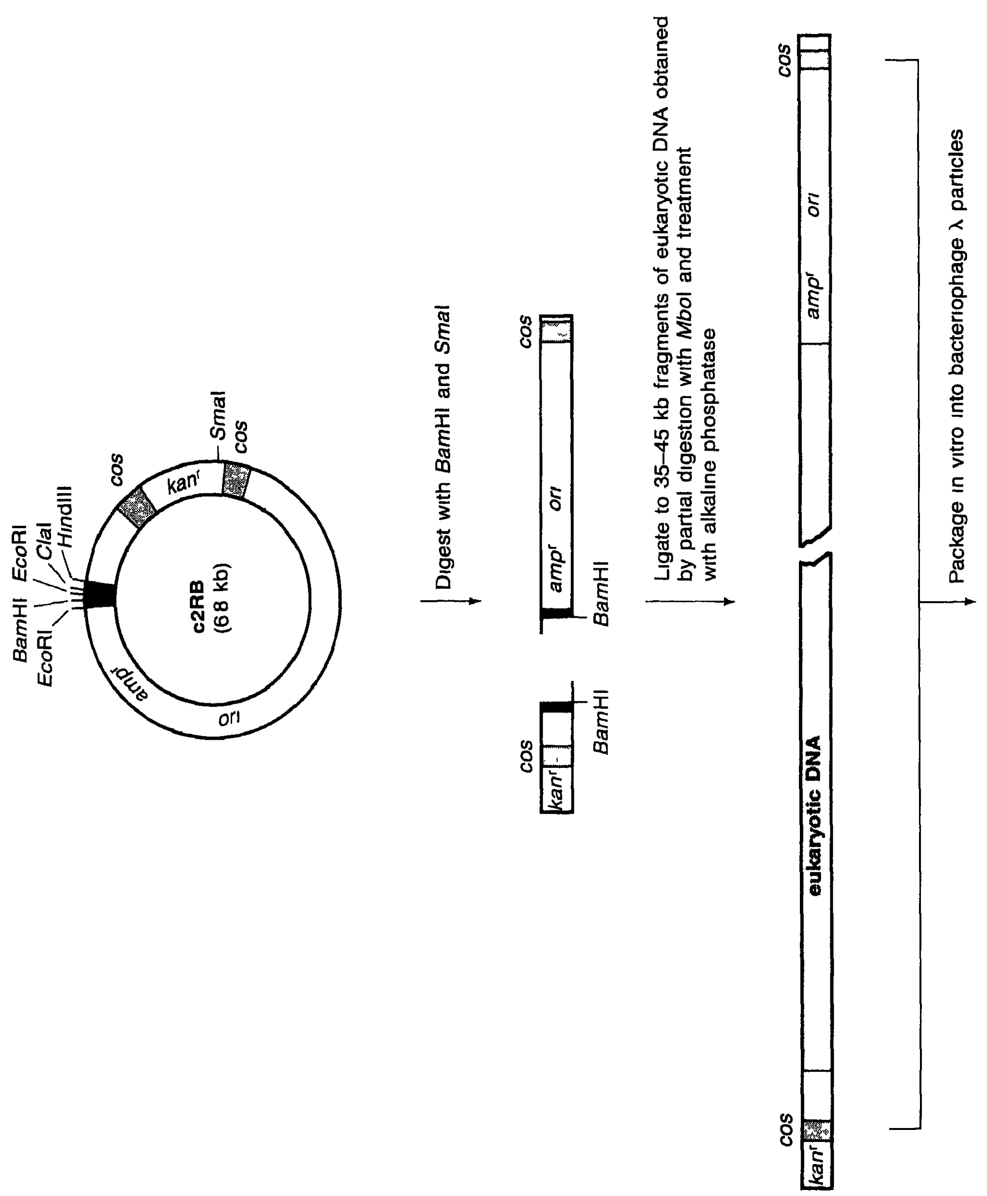
Cloning in cosmid vectors digested with two restriction enzymes This procedure is a modification of the protocol of Ish-Horowicz and Burke (1981) Following digestion of pJB8 DNA with *Hin*dIII or *Sal*I and inactivation of the protruding termini (e g , by alkaline phosphatase), the linear vector DNAs are cleaved with *Bam*HI The two vector fragments containing the *cos* sequence are isolated and ligated to 35–45-kb fragments of eukaryotic DNA generated by partial digestion with *Mbo*I or *Sau*3AI Note that an entire complement of plasmid sequences is contained between the two *cos* sites The concatemers are used as substrates for in vitro packaging of bacteriophage λ particles Following introduction into *E coli,* the cosmid DNA recircularizes and replicates in the form of a large plasmid The plasmid contains a β-lactamase gene that confers resistance to ampicillin on the host bacterium


BamHI
EcoRI
EcoRI
ClaI
HindIII
6000
XorII
amp^r
cos
1000
kan^r
c2RB
(6.8 kb)
SmaI
cos
5000
ori
2000
PvuII
4000
AvaI
SalI
3000

RB

c2RB (Bates and Swift 1983) is 6.8 kb in length. It contains two *cos* sites, derived from the cosmid vector MUA-3 (Meyerowitz et al. 1980); ampicillin resistance and kanamycin resistance (*kan*^{r}) genes and a ColE1 origin, all derived from pKC7 (Rao and Rogers 1979); and four restriction sites (*Bam*HI, *Eco*RI, *Cla*I, and *Hin*dIII) that can be used for cloning Concatenation of the vector during ligation to foreign DNA can be suppressed either by treating the linearized vector with phosphatase (see page 3 28) or by using a modified form of directional cloning (see Figure 3 2) Because a 1.7-kb fragment containing a *cos* site is lost during packaging (see Figure 3.3), the vector should accept segments of foreign DNA whose lengths lie between 33 kb and 46.5 kb Furthermore, since the 1 7-kb fragment also carries a kanamycin resistance gene, none of the recombinant cosmids should confer resistance to the antibiotic upon the recipient cells Because kanamycin-resistant colonies can arise only if concatenation of the vector occurs during ligation, the conditions of the ligation reaction should be adjusted to minimize the number of such colonies. Libraries are constructed in c2RB as shown in Figure 3.3 The vector is prepared by sequential digestion with *Sma*I and a second restriction enzyme, for example, *Bam*HI. This creates two fragments, each carrying a *cos* site and each bearing a cohesive terminus and a blunt end The digested vector DNA is then ligated to phosphatase-treated segments of foreign DNA of the appropriate length that carry termini compatible with the cohesive termini created by *Bam*HI The presence of high concentrations of ATP (5 mM) in the ligation reaction inhibits ligation of blunt-ended DNA without affecting ligation of cohesive termini (Ferretti and Sgaramella 1981). The concatenated DNA is then packaged in vitro into bacteriophage λ particles that are used to infect a *recA*$^-$ strain of *E coli*.

As discussed above for pJB8, the two *Eco*RI sites flanking the single *Bam*HI of c2RB are particularly useful when "walking" from one recombinant cosmid to another



BamHI
EcoRI
EcoRI
ClaI
HindIII
cos
kan^r
SmaI
cos
c2RB
(6.8 kb)
amp^r
ori
Digest with BamHI and SmaI
cos
kan^r
BamHI
cos
amp^r
ori
BamHI
Ligate to 35–45 kb fragments of eukaryotic DNA obtained by partial digestion with MboI and treatment with alkaline phosphatase
cos
kan^r
eukaryotic DNA
amp^r
ori
cos
Package in vitro into bacteriophage λ particles

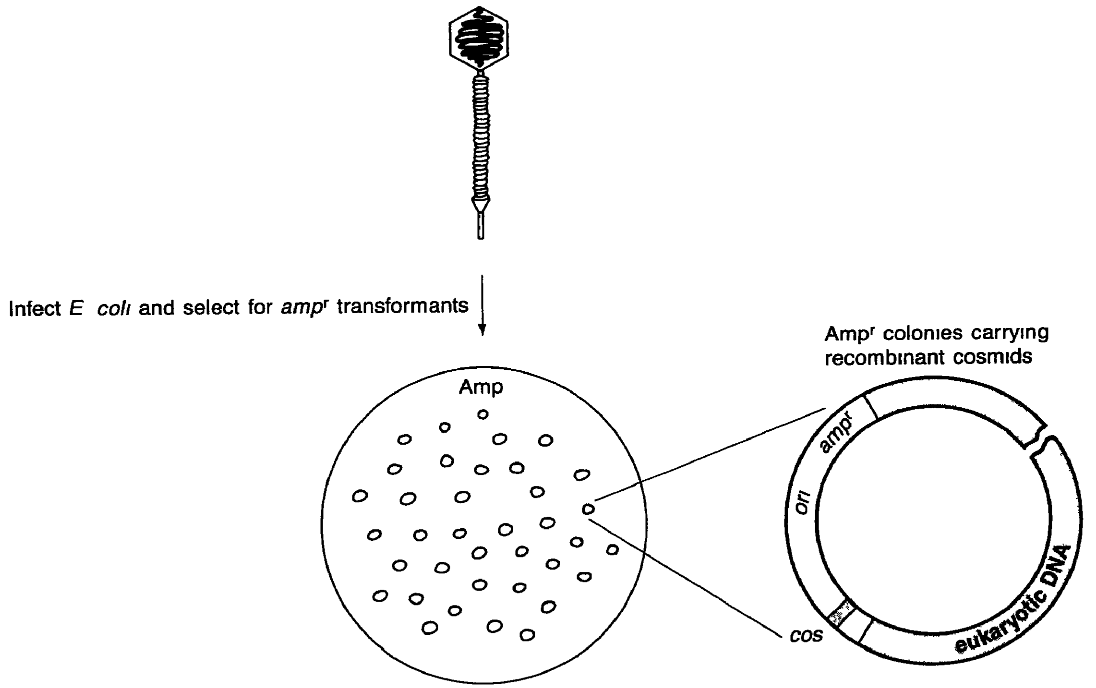
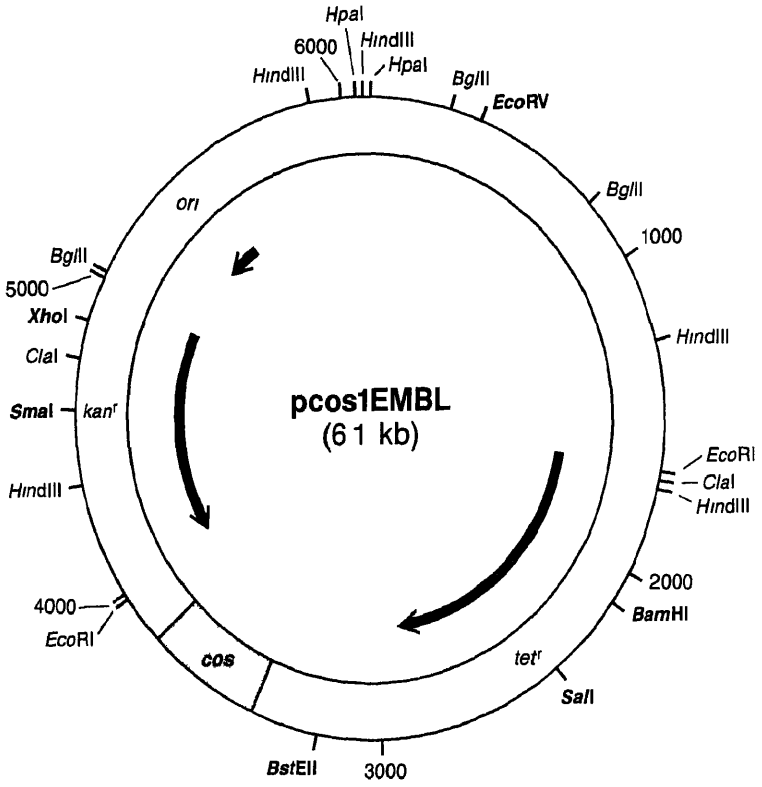

Infect E coli and select for amp^r transformants
Amp
Amp^r colonies carrying recombinant cosmids
amp^r
ori
cos
eukaryotic DNA

FIGURE 3.3

Cosmid cloning using double *cos* site vector c2RB (Bates and Swift 1983) Following digestion with *Bam*HI and *Sma*I, the cosmid DNA is ligated to dephosphorylated segments of foreign DNA of the appropriate length (35–45 kb) that carry termini compatible with *Bam*HI cohesive termini The concatenated DNA is then packaged into bacteriophage λ particles that are used to infect a *recA*$^-$ strain of *E coli*



HpaI
6000
HindIII
HindIII
HpaI
BglII
EcoRV
BglII
1000
HindIII
EcoRI
ClaI
HindIII
2000
BamHI
SalI
tet^r
3000
BstEII
cos
4000
EcoRI
HindIII
SmaI
kan^r
ClaI
XhoI
5000
BglII
ori
pcos1EMBL
(6 1 kb)

pcos1EMBL

pcos1EMBL (Poustka et al. 1984) contains a single *cos* site, derived from pHC79 (Hohn and Collins 1980); a tetracycline resistance (*tet*^{r}) gene, derived from pBR322; and a kanamycin resistance gene and an origin of replication, both derived from R6K (a plasmid that has no homology with plasmids carrying a ColE1 origin) (Kolter and Helinski 1978). This cosmid vector is used chiefly to screen libraries of recombinant cosmids by recombination in vivo (see Poustka et al. 1984). Libraries constructed in pcos1EMBL and established in a *recA*$^-$ host are packaged in vivo into bacteriophage λ particles. These particles are used to infect a *recA*$^+$ host that contains the target sequence cloned in a plasmid vector that carries an *amp*^{r} marker and that has no homology with pcos1EMBL. After recombination has occurred, cointegrants are packaged into bacteriophage λ particles and transferred to *recA*$^-$ recipients, which are selected for growth in the presence of both ampicillin and kanamycin. The essential features of this system are the lack of homology between the R6K-derived sequences and ColE1 and the compatibility of the R6K origin with the ColE1 origin. Recombinational screening with pcos1EMBL can therefore be carried out with any plasmid that contains a ColE1 origin and does not contain any portion of the tetracycline resistance gene of pBR322. Alternatively, it is possible to use a complementary recombinational screening system in which a cosmid vector built from a ColE1-derived plasmid is used (Most of the commonly used cosmid vectors belong to this class.) In this case, the screening plasmid carrying the target sequences is derived from a nonhomologous but compatible plasmid (see Lindenmaier et al 1985) Instead of constructing recombinant cosmid libraries in a vector like pcos1EMBL, it is therefore possible to carry out recombinational screening of existing libraries constructed in cosmid vectors built from ColE1-based plasmids.

COSMID VECTORS FOR TRANSFECTION OF MAMMALIAN CELLS

Several cosmid vectors have been developed to shuttle genes into and out of mammalian cells and to isolate eukaryotic genes by functional selection These vectors differ from one another chiefly in the prokaryotic and eukaryotic selectable markers they carry and in the restriction sites that can be used for cloning. Vectors of this class have been used as follows.

- *To introduce characterized recombinant cosmids containing particular genes of interest into eukaryotic cells* For example, Grosveld et al (1982) obtained expression of β-globin in L cells that had been transfected with a recombinant cosmid containing the human β-globin gene Other examples include the expression of α-globin in COS and L cells transfected with cosmids carrying the human α-globin gene cluster (Lau and Kan 1983) and the expression of tumor necrosis factor (TNF) in human fibrosarcoma cells transfected with autonomously replicating cosmids carrying the human locus coding for TNF (Kioussis et al 1987) In such experiments, the type of selectable marker and the presence of autonomous mammalian replicons within the cosmid vector determine the species and types of cells that can be used for transfection (See Chapter 16 for a detailed discussion of selectable markers for mammalian cells)
- *To facilitate selection of cells that have taken up DNA from a population transfected with a cosmid library* By applying the appropriate selection, cells that fail to incorporate cosmid DNA can be eliminated, leaving a manageable number of clones that subsequently can be screened by another procedure This approach was used by Lau and Kan (1984) to isolate a 43 8-kb DNA fragment that contains the human thymidine kinase (*tk*) gene A library of human genomic DNA was prepared in a cosmid vector (pCV108, see page 3 19) that carries a neomycin resistance (*neo*^{r}) gene DNA from the total library was used to transfect mouse L cells deficient in thymidine kinase, and cells that were able to survive in HAT medium (hypoxanthine/aminopterin/thymidine, see Chapter 16) were selected These cells were then checked for resistance to neomycin, and the cosmid clone was isolated by packaging genomic DNA in vitro (Lindenmaier et al 1982, Lund et al 1982, Lau and Kan 1984) Other schemes for identifying transformants that contain the desired recombinant cosmids might include screening for surface markers (Kavathas and Herzenberg 1983) or the acquisition of a visible phenotypic property such as transformation (Shih et al 1979)

Some cosmids that carry selectable markers for eukaryotic cells are described on the following pages.

pHC79-2*cos/tk*

pHC79-2*cos/tk* (Lindenmaier et al. 1982) is *amp*^{r} *tet*^{r} *tk*$^{+}$ and contains a ColE1 origin of replication and two copies of *cos* oriented in tandem. This arrangement of *cos* sequences increases the efficiency with which cosmid DNA can be packaged in vivo into transducing particles. The only useful cloning site in pHC79-2*cos/tk* is the unique *Cla*I site, which can be ligated to foreign DNA that has been partially digested with *Taq*I.

pCV103, pCV107, pCV108

These are derivatives of pJB8 (*amp*^{r}, ColE1 origin) that carry selectable markers for eukaryotic cells on segments of DNA inserted at the *Bal*I site of pJB8 (Lau and Kan 1983) pCV103 contains the *E. coli* hypoxanthine-guanine phosphoribosyl transferase (*hgprt*) gene and the SV40 origin of replication, both derived from SV2-*gpt* (Mulligan and Berg 1980). pCV107 contains the mouse dihydrofolate reductase (*dhfr*) gene and the SV40 origin of replication, both derived from SV2-*dhfr* (Subramani et al. 1981) pCV108 contains the neomycin resistance gene and the SV40 origin of replication, both derived from SV2-*neo* (Southern and Berg 1982)

pTM, pMCS, pNNL

pTM (Grosveld et al 1982) is a derivative of pJB8 (*amp*^{r}, ColE1 origin) that carries a neomycin resistance gene, derived from plasmid AG60 (Colbère-Garapin et al. 1981), inserted at the *Bam*HI site of pJB8 pMCS is identical to pTM except that it carries a copy of the SV40 origin of replication. pNNL is similar in structure to pTM, except that it carries the *E coli* hypoxanthine-guanine phosphoribosyl transferase gene instead of the neomycin resistance gene

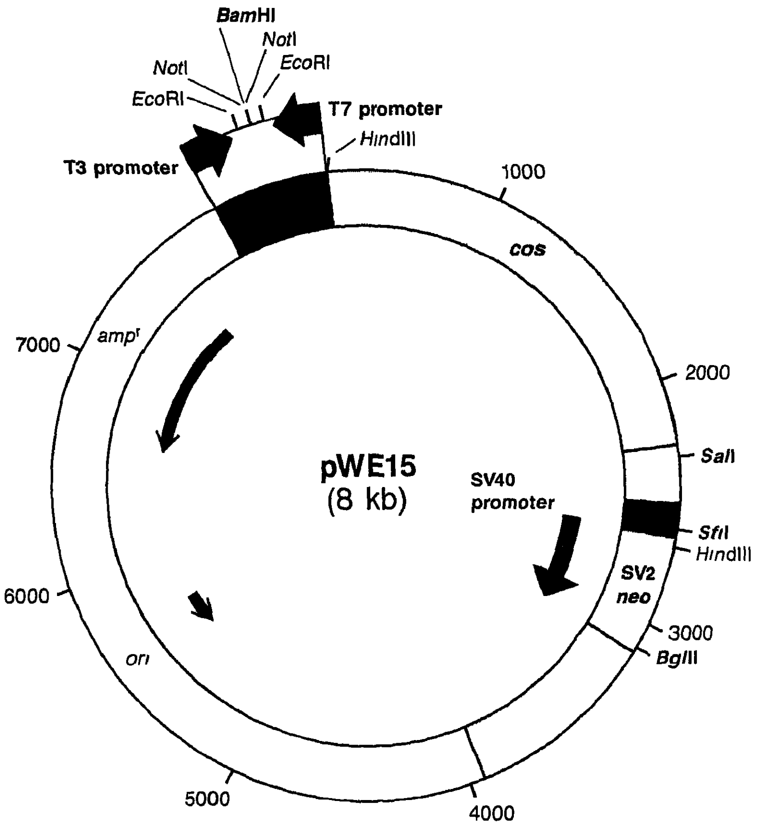
pHSG274

pHSG274 (Brady et al 1984) contains a kanamycin resistance gene that functions in both prokaryotes and eukaryotes and a ColE1 origin of replication Like pJB8, the *cos* cloning site in pHSG274 is flanked by restriction sites (*Sal*I and *Eco*RI) that allow directional cloning of foreign DNA

cos202, cos203

cos202 and cos203 (Kioussis et al. 1987) are approximately 10 kb in length They contain the Epstein-Barr virus origin of replication and the gene coding for the antigen EBNA-1 (Yates et al. 1985), which allow them to replicate autonomously in cultured human cells, the prokaryotic hygromycin B resistance (*hph*) gene (Gritz and Davies 1983) inserted between a eukaryotic promoter and polyadenylation signal (Sugden et al 1985); an ampicillin resistance gene and a ColE1 origin of replication, and a single *cos* site.

cos203 differs from cos202 in that it contains several restriction sites (*Hin*dIII, *Sal*I, *Xba*I, and *Bam*HI) that can be used for cloning segments of eukaryotic DNA between 28 kb and 42 kb in length


BamHI
NotI
EcoRI
NotI
EcoRI
T7 promoter
HindIII
T3 promoter
1000
cos
7000
amp^r
2000
SalI
pWE15
(8 kb)
SV40
promoter
SfiI
HindIII
SV2
neo
6000
3000
BglII
ori
5000
4000

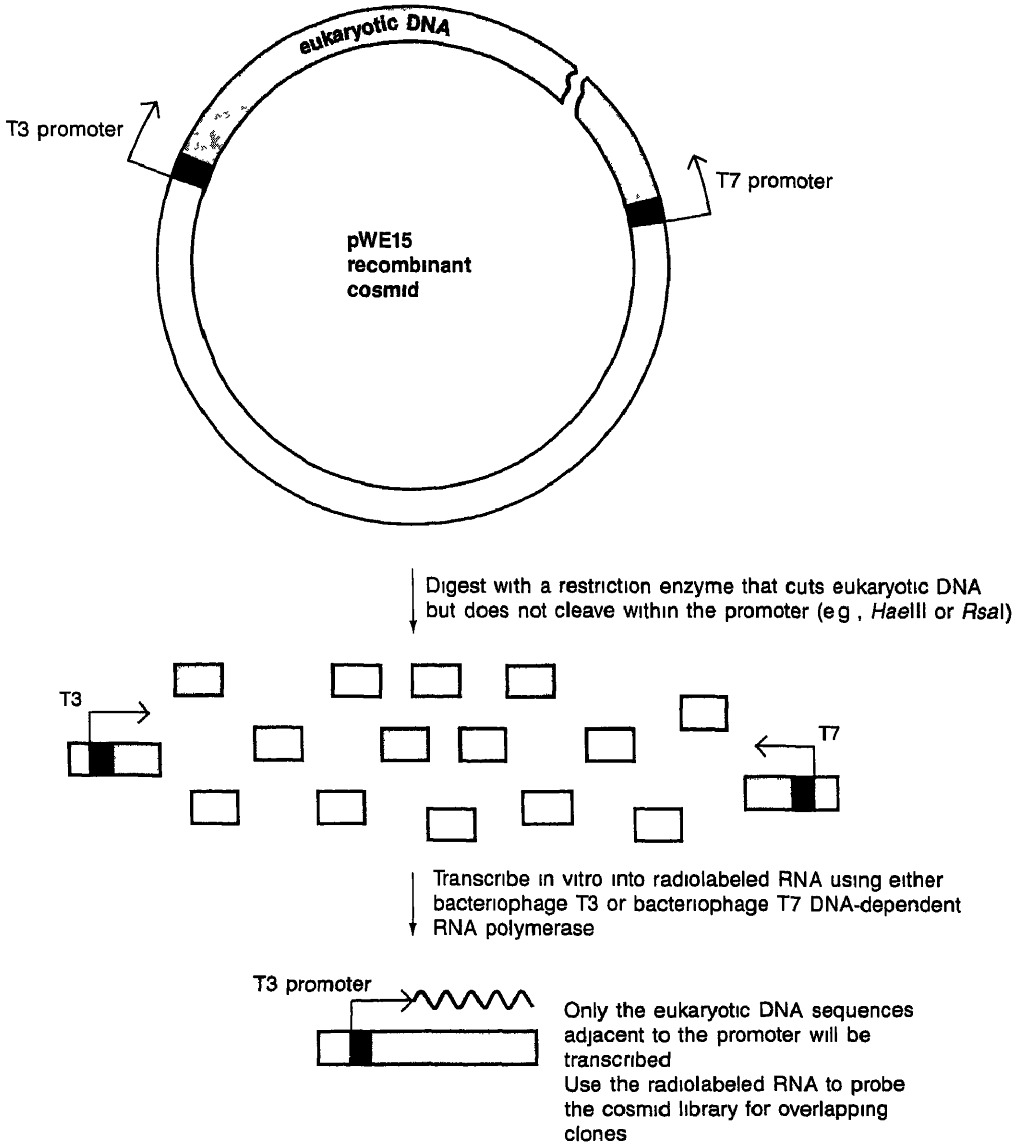
pWE15, pWE16

pWE15 and pWE16 (Wahl et al. 1987) were designed to simplify the process of "walking" from one cosmid clone to another. They are derivatives of cosmids pCV107 and pCV108 (Lau and Kan 1983) and contain bacteriophage T3 and T7 promoters on either side of recognition sites for the rarely cutting restriction enzyme *Not*I Fragments of foreign DNA are cloned into a *Bam*HI site lying between the two bacteriophage promoters pWE15 carries the neomycin resistance gene of Tn*5* in a form (SV2-*neo*) that can be expressed in mammalian cells, the selectable marker in pWE16 is the mouse dihydrofolate reductase gene. Both cosmids carry the SV40 origin of replication and sequences that enhance the expression of cloned genes

The bacteriophage T3 and T7 promoters form the basis of a simple method to prepare probes from the ends of large segments of DNA cloned in pWE15 and pWE16 These probes may then be used to identify overlapping clones in the cosmid library This "walking" process is diagrammed in Figure 3.4. The original cosmid is digested with a restriction enzyme, such as *Hae*III or *Rsa*I, that cleaves foreign DNA frequently but does not cleave within the bacteriophage T3 and T7 promoters Among the digestion products will be two fragments, each consisting of a small segment of foreign DNA positioned downstream from a bacteriophage promoter The entire population of DNA fragments is used as a template for either of the bacteriophage DNA-dependent RNA polymerases in the presence of radiolabeled rNTPs (see Chapter 10) Only those foreign sequences that are linked to the bacteriophage promoter will be transcribed into radiolabeled RNA Because the first nucleotide added by either polymerase is only four nucleotides from the site at which the foreign DNA is inserted, the RNA consists almost entirely of sequences that are derived from the termini of the original cloned segment of foreign DNA It can therefore be used as a probe to rescreen the cosmid library for overlapping clones In theory, problems can arise if the termini of the cloned segment contain sequences that are repeated many times in the original genome However, this problem is minimized if restriction enzymes are used that cleave the foreign DNA into pieces that are much smaller than the average distance between repeats.

The *Not*I recognition sites flanking the cloning site permit the excision of most inserts of foreign DNA as a single restriction fragment *Not*I recognizes an 8-bp sequence that consists only of G C base pairs and contains two CpG dinucleotides This sequence occurs very infrequently in mammalian DNA (about once in every 1500 kb) In almost all cases, therefore, the cloned segment of DNA will be excised as a single fragment. This can be advantageous for a number of reasons, for example, to simplify the construction of restriction maps or to facilitate removal of vector sequences prior to injection into fertilized mouse eggs to create lines of transgenic mice carrying the sequences of interest.

Note: Other vectors belonging to this series (e.g., pWE2, pWE4, pWE8, and pWE10, see Wahl et al. 1987) contain polycloning sites rather than a single *Bam*HI site. RNAs transcribed from these vectors contain at their 5′ termini a minimum of 30 nucleotides copied from the sequences of the polycloning sites that flank the inserted segments of foreign DNA. Although such vectors allow greater flexibility in cloning, the transcribed leader sequences can cause significant background hybridization when radiolabeled RNA is used to rescreen a cosmid library.



Digest with a restriction enzyme that cuts eukaryotic DNA but does not cleave within the promoter (e.g., *Hae*III or *Rsa*I)

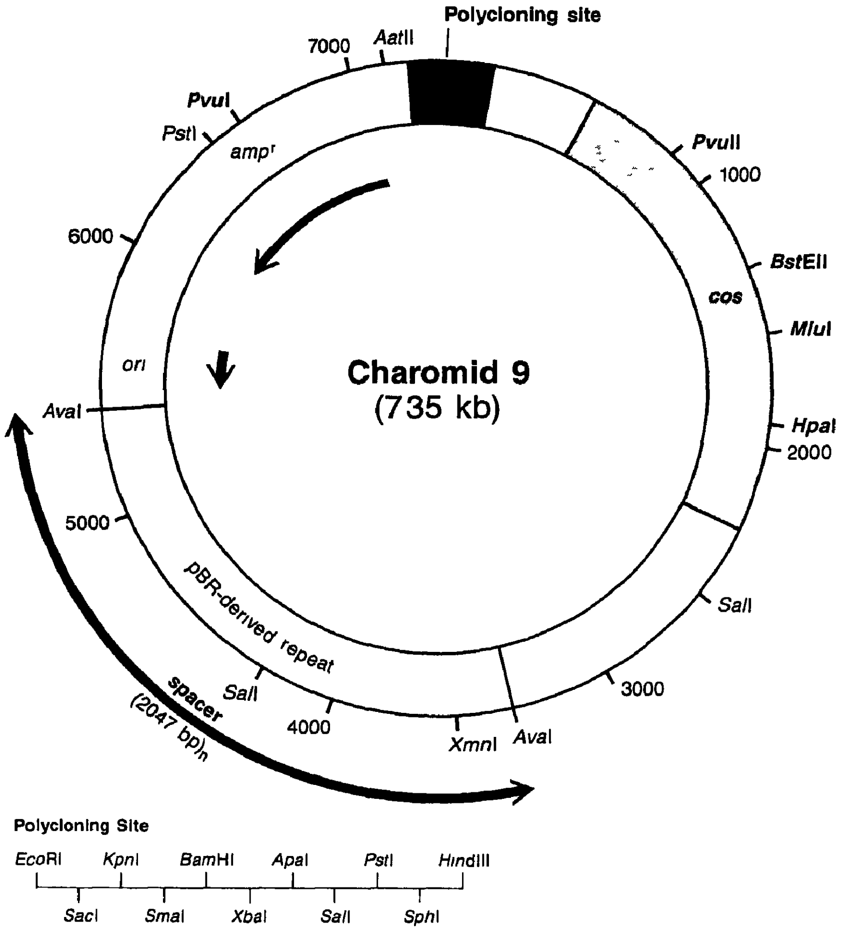
Transcribe in vitro into radiolabeled RNA using either bacteriophage T3 or bacteriophage T7 DNA-dependent RNA polymerase

Only the eukaryotic DNA sequences adjacent to the promoter will be transcribed

Use the radiolabeled RNA to probe the cosmid library for overlapping clones

FIGURE 3.4

Chromosome walking The figure shows in diagrammatic form the steps involved in chromosome walking using RNA probes generated by in vitro transcription of recombinant cosmids carrying promoters for bacteriophage-encoded DNA-dependent RNA polymerases



The *Eco*RI site in the polycloning site is adjacent to the *Aat*II site

CHAROMID 9 VECTORS

Charomid vectors (Saito and Stark 1986) were designed to extend the cloning range of cosmids to include DNA fragments that are shorter than 33 kb. They are a family of vectors built from a small (~5.3 kb) cosmid that contains the *cos* region from Charon 4A; an ampicillin resistance gene and a ColE1 origin of replication, both derived from pTBE (Grosveld et al. 1982); a polycloning site containing recognition sites for nine restriction enzymes (*Aat*II in the pBR322-derived sequence, followed by *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Apa*I, and *Hin*dIII in the polycloning site); and a spacer region consisting of 1–23 tandemly repeated copies of a 2-kb fragment derived from pBR322 and including the tetracycline resistance gene Variation in the length of the spacer region determines the size of foreign DNA that can be inserted into the vector and packaged into bacteriophage λ particles Because the spacer consists of head-to-tail repeats, individual charomid vectors carrying spacers of different sizes must be propagated in *recA*$^-$ bacteria. Transfer to *recA*$^+$ cells destabilizes the spacer and leads to the generation of charomids whose sizes range from 5.3 kb (no spacer) to 52 kb (23 tandem repeats, arranged in 2-kb intervals)

There are a number of potential uses for charomid vectors, including construction of genomic libraries of mammalian DNA that has been digested with any of a variety of restriction enzymes, singly or in combination, and cloning of specific fragments of genomic DNA that have been previously identified by Southern hybridization The ability of different members of the charomid family to accommodate a wide size range of fragments that have been generated by a large number of restriction enzymes provides great latitude in cloning strategy.

In addition to their usefulness in the cloning and mapping of fragments of genomic DNA, charomid vectors may turn out to be useful for other purposes (e g , the introduction of multiple copies of a gene into mammalian cells by transfection) However, until charomids become more widely adopted, their utility for these more specialized tasks and their ability to propagate genomic DNA fragments in a stable fashion will not be known.

Notes

Charomid vectors contain a *Sal*I site in each unit of the tandemly repeated sequence and a *Sal*I site in the polycloning site that is distal to the cloning site Digestion of a recombinant charomid with *Sal*I, followed by recircularization, therefore yields a replication-competent plasmid (with an ampicillin resistance gene) containing the entire segment of foreign DNA but lacking the spacer region This strategy of reducing the size of recombinant clones can greatly simplify tasks such as the construction of restriction maps of the foreign DNA However, it can only be carried out if the foreign DNA lacks a *Sal*I site Fortunately, the hexanucleotide sequence recognized by *Sal*I contains a CpG dinucleotide, and the enzyme therefore cleaves mammalian DNA relatively infrequently (about once in every 100 kb)

Charomid vectors contain a unique *Pvu*I site in the ampicillin resistance gene and a unique *Mlu*I restriction site within the region derived from Charon 4A that contains the *cos* site Both the *Pvu*I and *Mlu*I recognition sequences contain two CpG dinucleotides, and the two enzymes therefore cleave mammalian DNA extremely infrequently (about once in every 300 kb and 500 kb, respectively) The locations of these sites within the charomid allow restriction maps of foreign DNA to be generated rapidly by partial digestion. For example, digestion of a recombinant charomid by *Mlu*I yields a linear molecule that can be radiolabeled at both ends. The foreign DNA lies close to one end of the molecule and the other end is occupied by the tandemly arranged spacer A crude restriction map of the foreign DNA can therefore be constructed by measuring the sizes of the radioactive bands obtained after partial digestion of the linearized charomid with enzymes that do not cleave within the spacer (Smith and Birnstiel 1976) All radiolabeled fragments smaller than the spacer must be derived from the end of the charomid that carries the foreign DNA (Saito and Stark 1986) In a variation of this mapping strategy, the bacteriophage λ *ter* function was used to linearize the recombinant charomid, and the partial digestion products could be detected by their ability to form hybrids, using a radiolabeled oligonucleotide complementary to the cohesive termini as a probe (Rackwitz et al 1984, 1985).

Construction of Genomic DNA Libraries in Cosmid Vectors

Many of the procedures used to construct genomic DNA libraries in bacteriophage λ vectors also apply to cloning in cosmid vectors. In both systems, large segments of eukaryotic DNA, generated by quasi-random fragmentation, are ligated to vector DNA to form concatemers that can be packaged into bacteriophage λ particles. As discussed previously, libraries constructed in bacteriophage λ vectors are stored and propagated in the form of such recombinant bacteriophage particles. In cosmid cloning, however, these particles merely serve as vehicles by which the recombinant DNA molecules are efficiently introduced into bacteria, where they are propagated as large plasmids. If each bacterium in the population carries a different recombinant cosmid, about 350,000 transformants must be generated to achieve a 99% probability that a particular single-copy sequence of eukaryotic DNA will be represented in the library (see equation in Chapter 9, page 9.3). The cosmid library can then be either maintained as a population of bacteria (as a pool or as individual colonies on filters) or rescued by transduction and maintained as a stock of transducing bacteriophage particles.

The current method of choice for constructing libraries of eukaryotic genomic DNA in cosmid vectors is to clone DNA fragments of an appropriate size (usually 35–45 kb) that are obtained by partial digestion of high-molecular-weight DNA with a restriction enzyme (or enzymes) that recognizes a 4-bp sequence and generates a cohesive terminus compatible with that of the vector. The best enzymes for this purpose are *Mbo*I and *Sau*3AI, although *Taq*I or *Eco*RI* activity can also be used. In the examples given below, genomic DNA partially digested with *Mbo*I or *Sau*3AI is cloned into the *Bam*HI site of the cosmid pJB8. Three methods are described to suppress self-ligation and packaging of the vector DNA. In the first protocol (Meyerowitz et al. 1980; Grosveld et al. 1981), the linearized vector DNA is simply treated with phosphatase; in the second protocol (Ish-Horowicz and Burke 1981), which is more efficient but involves more enzymatic steps, a modified form of directional cloning is used. Finally, a modification of the second procedure is described that permits the construction of large cosmid libraries when limited amounts of genomic DNA are available.

CLONING IN PHOSPHATASE-TREATED COSMID VECTORS

The basic steps of this procedure are shown in Figure 3.5.

Preparation of Vector DNA

1. Digest 20 μg of closed circular pJB8 DNA, prepared according to the methods given in Chapter 1, with a two- to threefold excess of *Bam*HI for 1 hour. Remove an aliquot (0.3 μg) and analyze the extent of digestion by electrophoresis through a 0.8% agarose gel, using as a marker 0.3 μg of undigested pJB8 DNA. If digestion is incomplete, add more restriction enzyme and continue the incubation.

2. When digestion is complete, extract the sample with phenol:chloroform and precipitate the DNA with 2 volumes of ethanol for 15 minutes at 0°C. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge, and redissolve it in 180 μl of 10 mM Tris·Cl (pH 8.3). Remove a 4-μl aliquot (~0.5 μg) and store at −20°C.

3. To the remainder of the sample, add 20 μl of 10 × CIP dephosphorylation buffer and 0.25 unit of calf intestinal alkaline phosphatase (CIP). Incubate the reaction for 30 minutes at 37°C.

   10 × CIP dephosphorylation buffer

   10 mM $ZnCl_2$
   10 mM $MgCl_2$
   100 mM Tris·Cl (pH 8.3)

4. Add EDTA (pH 8.0) and SDS to final concentrations of 5 mM and 0.5%, respectively. Add proteinase K to a final concentration of 50 μg/ml. Incubate the reaction for 30 minutes at 56°C.

   Proteinase K is used to digest the CIP, which must be completely removed if the subsequent ligation reactions are to work efficiently. An alternative method to inactivate the CIP is to heat the reaction (at the end of step 3) to 65°C for 1 hour (or 75°C for 10 minutes) in the presence of 5 mM EDTA and then to extract once with phenol:chloroform.

5. Cool the reaction to room temperature, and extract once with phenol and once with phenol:chloroform. Add 0.1 volume of 3 M sodium acetate (pH 7.0). Mix well, and add 2 volumes of ethanol. Mix well, and store at 0°C for 15 minutes. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol at 4°C and recentrifuge.

6. Redissolve the DNA in 20 μl of TE (pH 7.6) and carry out a test ligation as described below to determine the effectiveness of the phosphatase treatment.

7. If the results of the test ligation are satisfactory, dispense the dephosphorylated DNA into 3-μg aliquots and store at −20°C.

Notes

i. The pH of the 3 M sodium acetate solution is 7 0 rather than the usual 5.2. At acid pH, EDTA precipitates from solution if its concentration exceeds 5–10 mM

ii. Until recently, CIP was supplied as a suspension in a slurry of ammonium sulfate. However, it may now be obtained as a stabilized solution (in 30 mM triethanolamine buffer [pH 7 6], 3 mM NaCl, 1 mM $MgCl_2$, 0 1 mM $ZnCl_2$) It should be stored undiluted in this buffer (not frozen).

TEST LIGATION

1. Set up the following three reaction mixtures

a 0.5 μg of phosphatase-treated *Bam*HI-pJB8 DNA

b. 0.5 μg of phosphatase-treated *Bam*HI-pJB8 DNA plus 0 5 μg of bacteriophage λ DNA cleaved to completion with *Bam*HI.

c 0 5 μg of *Bam*HI-pJB8 DNA *not* treated with phosphatase (i.e , the aliquot set aside in step 2, page 3.28)

2. To each mixture add:

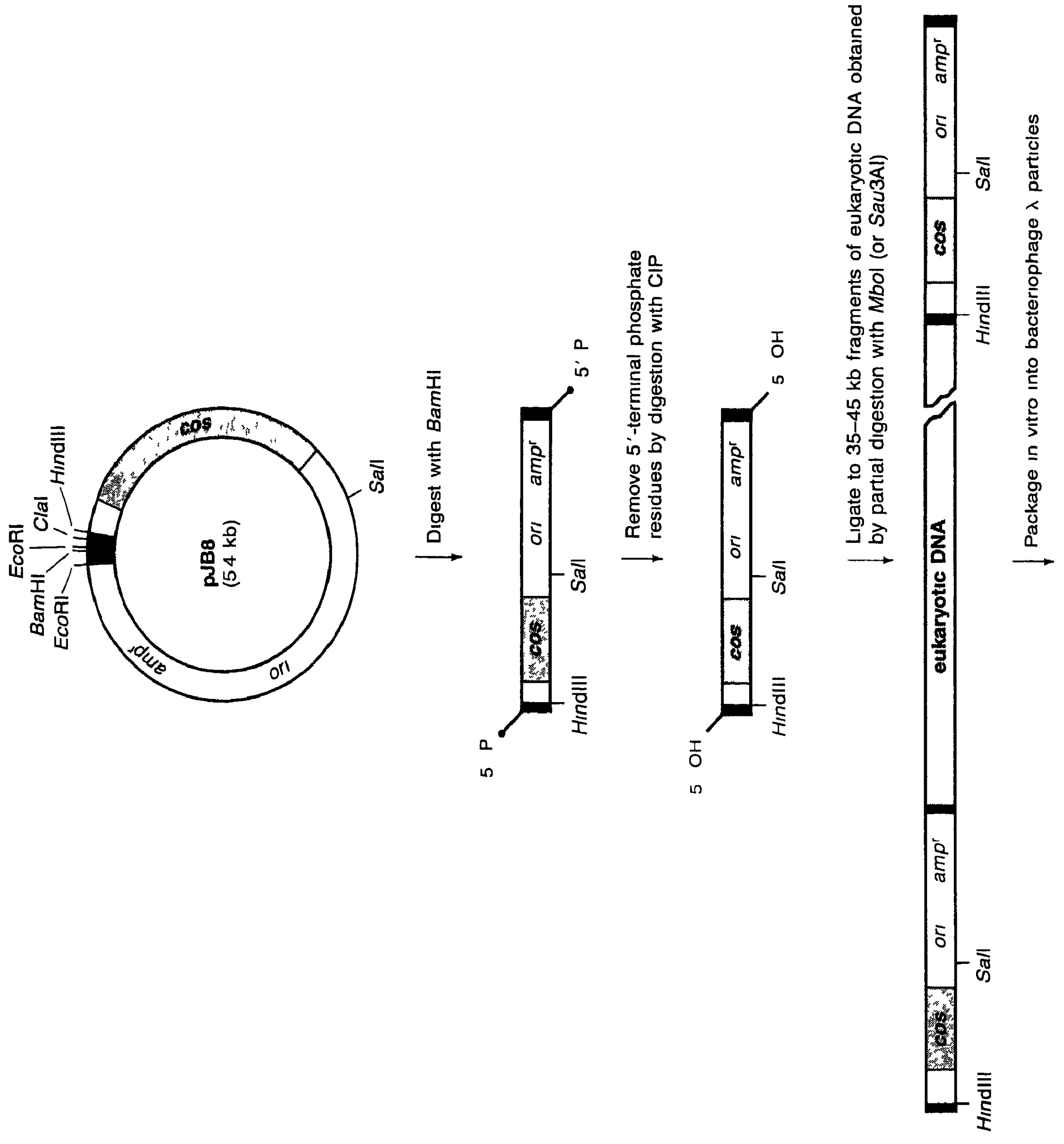
10 × ligation buffer	1 μl
10 mM ATP	1 μl
H_2O to 10 μl	

10 × Ligation buffer

0.5 M Tris · Cl (pH 7.6)
100 mM $MgCl_2$
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

3. Remove a 2-μl aliquot from each mixture and store at 4°C. Add 0 5 Weiss unit of bacteriophage T4 DNA ligase to the remainder of each mixture and incubate for 4–8 hours at 16°C. (For definition of Weiss unit, see page 3 33) Remove another 2-μl aliquot from each reaction, and assay all six aliquots by electrophoresis through a 0 7% agarose gel. Store the remainders of the reactions at −20°C

The phosphatase-treated sample (a) should show no evidence of ligation, whereas most of the linear vector DNA in the untreated sample (c) should have been converted into multimers. Because dephosphorylated, single-stranded protruding termini of DNA can be ligated to complementary cohesive termini that carry a 5′ phosphate, at least some of the phosphatase-treated cosmid DNA should have become ligated to the fragments of bacteriophage λ DNA that had been cleaved with *Bam*HI (b).


EcoRI
BamHI
EcoRI
ClaI
HindIII
cos
SalI
amp^r
ori
pJB8
(5.4 kb)
Digest with BamHI
5′ P
5′ P
cos
ori
amp^r
HindIII
SalI
Remove 5′-terminal phosphate residues by digestion with CIP
5′ OH
5′ OH
cos
ori
amp^r
HindIII
SalI
Ligate to 35–45 kb fragments of eukaryotic DNA obtained by partial digestion with MboI (or Sau3AI)
cos
ori
amp^r
eukaryotic DNA
cos
ori
amp^r
HindIII
SalI
HindIII
SalI
Package in vitro into bacteriophage λ particles

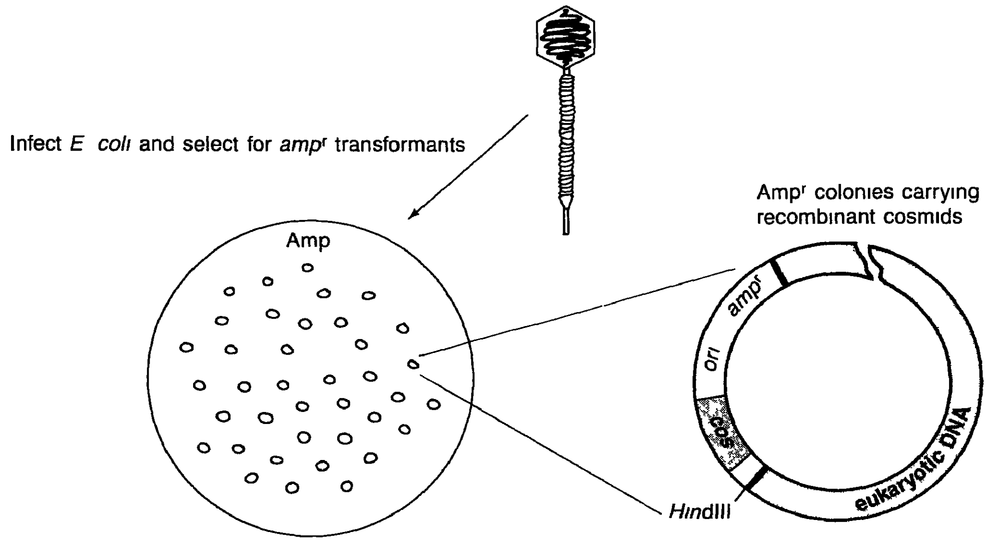

Infect E coli and select for amp^r transformants
Amp
Amp^r colonies carrying recombinant cosmids
amp^r
ori
cos
eukaryotic DNA
HindIII

FIGURE 3.5

Cloning in phosphatase-treated vectors DNA of the cosmid pJB8 is digested with *Bam*HI and dephosphorylated with alkaline phosphatase to yield a vector with protruding 5′ termini that can be ligated to 35–45-kb fragments of eukaryotic DNA generated by partial digestion with *Mbo*I or *Sau*3AI The resultant concatemers serve as substrates for in vitro packaging of bacteriophage λ particles Following introduction into *E coli*, the cosmid DNA recircularizes and replicates in the form of a large plasmid The plasmid contains a β-lactamase gene that confers resistance to ampicillin on the host bacterium

Partial Digestion of Eukaryotic DNA with MboI or Sau3AI

Prepare the eukaryotic DNA for cloning by partial digestion with *Mbo*I or *Sau*3AI, using the procedures described in detail in Chapter 9 Because large fragments of DNA are required for cloning in most cosmid vectors, the eukaryotic DNA must be at least 200 kb in size before digestion Following partial restriction endonuclease digestion, select DNA in the size range appropriate for the vector (usually 35–45 kb) by sedimentation through a sucrose density gradient as described in Chapter 2, page 2 85 Intact bacteriophage λ DNAs of various sizes or multimers of pBR322 should be used as markers

Ligation and Packaging

The total concentration of DNA in the ligation reaction should be greater than 200 μg/ml to favor the formation of mixed concatemers between the arms of the cosmid vector and the eukaryotic DNA. Because of the inability of the vector to self-ligate, the reaction can be driven with a molar excess of vector molecules. The following ligation reactions contain a 9:1 molar ratio of vector DNA:eukaryotic DNA.

1. Mix.

phosphatase-treated vector DNA	3 μg
fragments of eukaryotic DNA (35–45 kb in size)	3 μg
10 × ligation buffer (see page 3.29 for recipe)	2 μl
10 mM ATP	2 μl
H_2O to 20 μl	

Remove an aliquot (1 μl) and store at 4°C. Add 1–2 Weiss units of bacteriophage T4 DNA ligase to the remainder of the reaction and incubate overnight at 16°C.

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

2. At the end of the ligation reaction, remove another 1-μl aliquot and analyze it by conventional electrophoresis through a 0.4% agarose gel or by pulsed-field gel electrophoresis (see Chapter 6) together with the aliquot set aside in step 1. If the ligation has been successful, some of the eukaryotic DNA should have been converted to high-molecular-weight concatemers. Most of the vector DNA should remain unligated.

3. Package the ligated DNA into bacteriophage λ particles, as described in Chapter 2, page 2.95, using no more than 0.5 μg of concatenated DNA per packaging reaction. As controls, package the samples set aside in step 3 of the test ligation, page 3.29.

The size of the inserts that can be cloned in cosmids is affected by the method used to prepare packaging extracts (Bates and Swift 1983). Extracts used to package cosmids should be prepared using a buffer that contains spermidine but not putrescine. When putrescine is omitted from the packaging extract and the packaging reaction, the system exhibits selectivity in the size of DNA molecules that are packaged. Thus, DNA that is 80% of wild-type bacteriophage λ DNA in length is packaged 200-fold less efficiently than wild-type bacteriophage λ DNA itself. In the absence of putrescine, cosmids that contain large inserts will be preferentially packaged. For unknown reasons, possibly because of a shortage of *ter* function, some preparations of packaging extracts that work well with bacteriophage λ DNA are not suitable for packaging cosmids (F. Bass, pers. comm.). It is therefore advisable to test each batch of extracts with the products of a standardized ligation reaction between cosmid DNA and size-selected eukaryotic DNA.

4. After packaging is complete, add 0.5–1.0 ml of SM to each reaction Store the reactions at 4°C Mix a 10-μl aliquot of each reaction with 0 1 ml of SM and 0.2 ml of a fresh overnight culture of a *recA*$^-$ strain of *E coli* (e g , ED8767 or DH1) grown in Terrific Broth (TB) in the presence of 10 mM $MgCl_2$ and 0.2% maltose

   ED8767 and DH1 are less "leaky" *recA*$^-$ strains than HB101 and may therefore more effectively suppress recombination between repetitive sequences of cloned eukaryotic DNA

   Allow the bacteriophage particles to adsorb by incubating the infected cultures for 20 minutes at 37°C Then add 1 ml of TB and continue the incubation for a further 45 minutes at 37°C to allow expression of the ampicillin resistance gene.

   The ability of the plating bacteria to be infected efficiently with bacteriophage λ should be verified before they are used to establish the cosmid library This is best done by measuring the titer of a known bacteriophage stock on the plating bacteria

5. Spread 0.5 ml and 0.1 ml of the bacterial culture onto TB agar plates containing ampicillin (50 μg/ml) After incubating the plates overnight at 37°C, count the number of bacterial colonies Each microgram of ligated cosmid–eukaryotic DNA should yield between 5×10^4 and 5×10^5 bacterial colonies.

6. Pick a number of individual colonies and grow small-scale (5-ml) overnight cultures in TB containing 50 μg/ml ampicillin Isolate the cosmid DNA from 4–5 ml of each bacterial culture, using the alkaline lysis method (see Chapter 1, page 1.25) Digest the plasmid DNA with restriction enzyme(s) and analyze the sizes of the resulting fragments by gel electrophoresis

CLONING IN COSMID VECTORS DIGESTED WITH TWO RESTRICTION ENZYMES AND TREATED WITH PHOSPHATASE

Ish-Horowicz and Burke (1981) have described a modified procedure for the preparation of cosmid vectors such as pJB8 that carry a single *cos* site This procedure substantially reduces the possibility of obtaining recombinant clones containing multiple copies of the vector. The principle of the method is shown in Figure 3.2. In two separate aliquots, the cosmid vector is digested with restriction enzymes (*Hin*dIII and *Sal*I) that cleave on either side of the *cos* site. The single-stranded protruding termini of the resulting molecules are rendered incapable of ligation by any one of several enzymatic treatments (alkaline phosphatase is the one most commonly used, but nuclease S1 and mung bean nuclease also work) It is this treatment that suppresses the formation of tandem vectors during ligation and reduces the number of bacterial colonies that contain cosmids but no inserts The linear DNA molecules are then digested with *Bam*HI, and the fragments containing the *cos* sequence are isolated by gel electrophoresis. These fragments are ligated to fragments of eukaryotic DNA generated by partial cleavage with *Mbo*I or *Sau*3AI to form simple concatemers that serve as substrates in the packaging reaction. The destruction of the single-stranded protruding termini created by the first enzyme prevents the formation of more complex concatemers

In their original procedure, Ish-Horowicz and Burke did not select DNA fragments of a given size to insert into the cosmid vector Instead, high-molecular-weight eukaryotic DNA was digested with *Mbo*I until the average size of the digestion products was 35–45 kb The DNA was then dephosphorylated by treatment with alkaline phosphatase to prevent the joining together of smaller fragments of DNA into molecules large enough to be packaged into bacteriophage λ particles In most circumstances, we recommend including a sizing step and omitting the alkaline phosphatase treatment These changes improve the efficiency of ligation and reduce the potential for production of cosmids carrying sequences of DNA that are noncontiguous in the original eukaryotic genome If the amount of eukaryotic DNA is too small to allow fractionation by size, use the procedure originally described by Ish-Horowicz and Burke (1981) (see page 3.36)

The major disadvantages of the Ish-Horowicz and Burke procedure are the large number of enzymatic steps and the necessity to purify the arms of the cosmid vector by gel electrophoresis To avoid these problems, Bates and Swift (1983) and Poustka et al (1984) have constructed vectors (e g , c2RB) that greatly simplify the preparation of cosmid vector arms Their approach is outlined in Figure 3 3 and is described in detail on pages 3 42–3 43

Preparation of Vector DNA

1. Digest an aliquot (20 μg) of closed circular pJB8 DNA, prepared according to the methods given in Chapter 1, with a two- to threefold excess of *Hin*dIII for 1 hour. At the same time, digest a second aliquot (20 μg) with *Sal*I. Remove an aliquot (0.3 μg) from each reaction, and analyze the extent of digestion by electrophoresis through a 0.8% agarose gel, using as a marker 0.3 μg of undigested pJB8 DNA. If digestion is incomplete, add more restriction enzyme and continue the incubation.

2. When the digestions are complete, extract the samples with phenol chloroform and precipitate the DNAs with 2 volumes of ethanol for 5 minutes at 0°C. Redissolve each sample of DNA in 180 μl of 10 mM Tris·Cl (pH 8.3). Remove a 4-μl aliquot (~0.5 μg) from each sample and store at −20°C.

3. To the remainders of the samples, add 20 μl of 10 × CIP dephosphorylation buffer and 0.25 unit of calf intestinal alkaline phosphatase (CIP). Incubate the reactions for 30 minutes at 37°C.

   10 × CIP dephosphorylation buffer

   10 mM $ZnCl_2$
   10 mM $MgCl_2$
   100 mM Tris·Cl (pH 8.3)

4. Add EDTA (pH 8.0) and SDS to final concentrations of 5 mM and 0.5%, respectively. Add proteinase K to a final concentration of 50 μg/ml. Incubate the reactions for 30 minutes at 56°C.

   Proteinase K is used to digest the CIP, which must be completely removed if the subsequent ligation reactions are to work efficiently. An alternative method is to heat the reaction (at the end of step 3) to 65°C for 1 hour (or 75°C for 10 minutes) and then to extract once with phenol chloroform.

5. Cool the reaction to room temperature, and extract once with phenol and once with phenol chloroform. Add 0.1 volume of 3 M sodium acetate (pH 7.0). Mix well, and add 2 volumes of ethanol. Mix well, and store at 0°C for 15 minutes. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol at 4°C and recentrifuge.

   Depending on the cosmid vector and the restriction enzymes used, other methods may be required to inactivate the cohesive termini of the vector. For example, because dephosphorylation of recessed 5′-phosphoryl termini is relatively inefficient, protruding 3′ termini should be removed by digestion with nuclease S1 or mung bean nuclease.

6. Redissolve each of the two samples of DNA in separate 50-μl aliquots of TE (pH 7.6). To test the effectiveness of the phosphatase treatment, set up a series of test ligations as described in step 1 on page 3.29. (Note that the bacteriophage λ DNA fragments in step 1, reaction *b*, should be

generated by digestion with *Hin*dIII or *Sal*I.) While carrying out the test ligations, store the remainders of the DNA samples at −20°C.

7. If the results of the test ligations are satisfactory (i.e., if no ligation of the vector is detected), thaw the DNA samples and add to each.

10 × *Bam*HI restriction enzyme buffer	10 μl
H_2O	40 μl
*Bam*HI	2–3-fold excess

Incubate the reactions for 1 hour at 37°C. Assay the extent of digestion by analyzing an aliquot of each reaction by electrophoresis through a 0.8% agarose gel.

8. When digestion is complete, separate the DNA fragments in the remainders of the samples by electrophoresis through a 0.8% agarose gel. Isolate the larger of the two *Bam*HI/*Hin*dIII DNA fragments and the smaller of the two *Bam*HI/*Sal*I DNA fragments by electrophoresis onto DEAE-cellulose membranes as described in Chapter 6, pages 6.24–6.27.

9. Redissolve the recovered DNA fragments in 20 μl of TE (pH 8.0). Estimate the amount of DNA in the samples by ethidium bromide fluorescence.

Notes

i. The pH of the 3 M sodium acetate solution is 7.0 rather than the usual 5.2. At acid pH, EDTA precipitates from solution if its concentration exceeds 5–10 mM.

ii. Until recently, CIP was supplied as a suspension in a slurry of ammonium sulfate. However, it may now be obtained as a stabilized solution (in 30 mM triethanolamine buffer [pH 7.6], 3 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$). It should be stored undiluted in this buffer (not frozen).

Treatment of Eukaryotic DNA with Alkaline Phosphatase

If the supply of eukaryotic DNA is plentiful, prepare it for cloning by partial digestion and fractionation according to size as described in Chapter 9. However, if the amount of DNA is limited, do not attempt to fractionate the DNA by density gradient centrifugation, since it is possible that insufficient DNA will be recovered. Under these circumstances, the best course of action is to digest the DNA to the point where its average size is approximately 35–45 kb and then to treat it with alkaline phosphatase. This inhibits the joining together of smaller fragments of DNA to form molecules of a size that could be packaged into bacteriophage λ particles (Ish-Horowicz and Burke 1981). If dephosphorylation is omitted or is carried out inefficiently, there is a strong risk that smaller fragments of eukaryotic DNA in the preparation will ligate to one another and to a cosmid vector, producing recombinants that contain sequences derived from two or more noncontiguous segments of the genome. This risk is only partially reduced by the constraints imposed by the in vitro packaging reaction. A population of DNA fragments with an average size of 42 kb will contain many molecules that are shorter than the mean. Although these shorter molecules may compose only a fraction of the total *weight* of the DNA, they make a more substantial contribution to the *number* of molecules in the population. Since the kinetics of ligation are determined by the concentration of reactive termini of DNA, these smaller molecules become preferentially incorporated into concatemers. This can be prevented only by dephosphorylation.

1. Using the procedures described in detail in Chapter 9, fragment 30 μg of eukaryotic DNA to a modal size of 45 kb by partial digestion with *Mbo*I or *Sau*3AI. Instead of fractionating the DNA by density gradient centrifugation, remove the 5′-phosphate residues by treatment with alkaline phosphatase as described below.

2. Heat the sample to 70°C for 15 minutes to inactivate the restriction enzyme, and then add $ZnCl_2$ to a final concentration of 1 mM and 2 units of calf intestinal alkaline phosphatase (CIP). Remove an aliquot (1 μg) of the reaction to a small (0.5-ml) microfuge tube containing 0.3 μg of a linearized plasmid (e.g., pUC cleaved with *Bam*HI). Set up a control that contains 0.3 μg of the same linearized plasmid in 10 μl of 1 × restriction enzyme buffer. Incubate all three samples for 30 minutes at 37°C.

3. To the large reaction, add SDS and EDTA (pH 8.0) to final concentrations of 0.5% and 5 mM, respectively. Add proteinase K to a final concentration of 50 μg/ml. Incubate the reaction for 30 minutes at 56°C.

   Proteinase K is used to digest the CIP, which must be completely removed if the subsequent ligation reactions are to work efficiently. An alternative method to inactivate the CIP is to heat the reaction (at the end of step 2) to 65°C for 1 hour (or 75°C for 10 minutes) and then to extract once with phenol:chloroform.

   Cool the reaction to room temperature, and carefully extract once with phenol and once with phenol:chloroform. Add 0.1 volume of 3 M sodium acetate (pH 7.0) and precipitate the DNA with 2 volumes of ethanol for 15 minutes at 0°C. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C. Wash the pellet with 70% ethanol at 4°C and recentrifuge. Allow the DNA to redissolve overnight at 4°C in 50 μl of TE (pH 7.6).

4. Meanwhile, heat the smaller (control) reactions to 75°C for 10 minutes (to inactivate the CIP) Cool the reactions to room temperature, and extract once with phenol and once with phenol:chloroform. Add 0.1 volume of 3 M sodium acetate (pH 7 0) and precipitate the DNAs with 2 volumes of ethanol for 15 minutes at 0°C Recover the DNAs by centrifugation at 12,000*g* for 10 minutes at 4°C Wash the pellets with 70% ethanol at 4°C and recentrifuge. Redissolve the DNAs in 10 μl of TE (pH 7.6).

5. Ligate the eukaryotic DNA (either dephosphorylated or size-selected) to the arms of the cosmid vector as described below

Notes

i The pH of the 3 M sodium acetate solution is 7.0 rather than the usual 5.2. At acid pH, EDTA precipitates from solution if its concentration exceeds 5–10 mM

ii Until recently, CIP was supplied as a suspension in a slurry of ammonium sulfate However, it may now be obtained as a stabilized solution (in 30 mM triethanolamine buffer [pH 7 6], 3 mM NaCl, 1 mM $MgCl_2$, 0 1 mM $ZnCl_2$). It should be stored undiluted in this buffer (not frozen)

Ligation and Packaging

The total concentration of DNA in the ligation reaction should be greater than 200 µg/ml to favor the formation of mixed concatemers between the arms of the cosmid vector and the eukaryotic DNA (see discussion in Chapter 1, pages 1.9–1.11). Because of the inability of the vector to self-ligate, the reaction can be driven with a molar excess of vector molecules The following ligation reactions contain a 9:1 molar ratio of vector DNA.eukaryotic DNA

1. Set up two reaction mixtures as follows.

Reaction a

fragments of eukaryotic DNA (35–45 kb in size)	2 µg
*Hin*dIII/*Bam*HI fragment of pJB8	1 µg
SalI/*Bam*HI fragment of pJB8	1 µg
10 × ligation buffer (see page 3 29 for recipe)	2 µl
10 mM ATP	2 µl
H_2O to 20 µl	

Reaction b

*Hin*dIII/*Bam*HI fragment of pJB8	1 µg
*Sal*I/*Bam*HI fragment of pJB8	1 µg
10 × ligation buffer	2 µl
10 mM ATP	2 µl
H_2O to 20 µl	

Remove an aliquot (2 µl) from each mixture and store at 4°C. Add 1–2 Weiss units of bacteriophage T4 DNA ligase to each mixture (For definition of Weiss unit, see page 3 33) Incubate the reactions overnight at 16°C

2. At the end of the ligation reactions, remove another 2-µl aliquot from each reaction and analyze them by pulsed-field gel electrophoresis or by electrophoresis through a 0.4% agarose gel together with the aliquots reserved in step 1. If the ligation has been successful, the eukaryotic DNA should have been converted to high-molecular-weight concatemers The arms of cosmid DNA should have been converted to homo- and heterodimers

It is important to see ligation of *both* vector DNA *and* eukaryotic DNA If no ligation of either species is seen, the presence of inhibitors in the preparation of eukaryotic DNA should be suspected In this case, extract the DNA again with phenol. chloroform and precipitate with ethanol If the arms of the vector can be ligated but the eukaryotic DNA cannot, it is likely that only a minority of the ends of the eukaryotic DNA carry the correct cohesive terminus

3. Carry out control experiments to check that the efficiency of the packaging mixtures exceeds 10^8 pfu/µg of bacteriophage λ DNA. Then package an aliquot of the ligated DNA into bacteriophage λ particles as described in Chapter 2, page 2 95, using no more than 0.5 µg of concatenated DNA in the packaging reaction. As controls, package the samples set aside in step 3 of the test ligation, page 3.29. Store the unused portion of the ligation mixture at 4°C.

The size of the inserts that can be cloned in cosmids is affected by the method used to prepare packaging extracts (Bates and Swift 1983). Extracts used to package cosmids should be prepared using a buffer that contains spermidine but not putrescine. When putrescine is omitted from the packaging extract and the packaging reaction, the system exhibits selectivity in the size of DNA molecules that are packaged. Thus, DNA that is 80% of wild-type bacteriophage λ DNA in length is packaged 200-fold less efficiently than wild-type bacteriophage λ DNA itself. In the absence of putrescine, cosmids that contain large inserts will be preferentially packaged. For unknown reasons, possibly because of a shortage of *ter* function, some preparations of packaging extracts that work well with bacteriophage λ DNA are not suitable for packaging cosmids (F. Bass, pers. comm.). It is therefore advisable to test each batch of extracts with the products of a standardized ligation reaction between cosmid DNA and size-selected eukaryotic DNA.

4. After packaging is complete, add 1 ml of SM to the packaging reactions. Store the reactions at 4°C. Mix 1-μl and 10-μl aliquots of the reactions with 0.1 ml of SM and 0.2 ml of a fresh overnight culture of a *recA*$^-$ strain of *E. coli* (e.g., ED8767 or DH1) grown in Terrific Broth (TB) in the presence of 10 mM $MgCl_2$ and 0.2% maltose.

   ED8767 and DH1 are less "leaky" *recA*$^-$ strains than HB101 and may therefore more effectively suppress recombination between repetitive sequences of cloned eukaryotic DNA.

   Allow the bacteriophage particles to adsorb by incubating the infected cultures for 20 minutes at 37°C. Then add 1 ml of TB and continue the incubation for a further 45 minutes at 37°C to allow expression of the ampicillin resistance gene.

   The ability of the plating bacteria to be infected efficiently with bacteriophage λ should be verified before they are used to establish the cosmid library. This is best done by measuring the titer of a known bacteriophage stock on the plating bacteria.

5. Spread 0.5 ml and 0.1 ml of the bacterial culture onto TB agar plates containing ampicillin (50 μg/ml). After incubating the plates overnight at 37°C, count the number of bacterial colonies. Each microgram of ligated cosmid–eukaryotic DNA should yield between 5×10^4 and 5×10^5 bacterial colonies. Calculate the total yield of cosmids in the original ligation mixture. If the number is satisfactory, package the remainder of the ligation mixture. Use no more than 0.5 μg of concatenated DNA per packaging reaction. Store the packaged cosmids in SM containing a few drops of chloroform until the library is amplified (see page 3.44) or screened (see Chapter 1).

6. Meanwhile, pick a number of individual colonies and grow small-scale (5-ml) overnight cultures in TB containing 50 μg/ml ampicillin. Isolate the cosmid DNA from 4–5 ml of each bacterial culture, using the alkaline lysis method (see Chapter 1, page 1.25). Digest the cosmid DNA with restriction enzyme(s) and analyze the sizes of the resulting fragments by gel electrophoresis.

PREPARATION OF VECTORS CONTAINING TWO *cos* SITES

Cosmid vectors that contain only one *cos* site (e g , pJB8 and its derivatives) are prepared by digestion with combinations of restriction enzymes and dephosphorylated by treatment with alkaline phosphatase Fragments of the vectors are then purified by gel electrophoresis Because of the complexity of these manipulations, the final yield of cosmid arms is often disappointingly low. To avoid these problems, Bates and Swift (1983) have described a simplified procedure for the preparation of the arms of a modified cosmid vector that contains two *cos* sites (see Figure 3 3, pages 3 14–3 15) In this vector, the *cos* sites are separated by the recognition site for a restriction enzyme that generates blunt ends. The vector arms can thus be easily prepared by simple digestion with two restriction enzymes that cleave at the cloning site and at the site between the two *cos* sequences When the mixture of arms is ligated to dephosphorylated eukaryotic DNA in the presence of high concentrations of ATP (5 mM), rejoining of the blunt ends occurs very inefficiently (Ferretti and Sgaramella 1981) Under these conditions, the major products of the ligation reaction are simple concatemers consisting of a segment of eukaryotic DNA bounded at either end by a cosmid arm The *cos* sites are oriented in the same manner in approximately 50% of the concatemers, which can therefore be packaged into bacteriophage λ particles In the protocol given below, the cosmid vector c2RB (Bates and Swift 1983) (see pages 3 12–3.13) is used as an example However, the procedure can easily be adapted to other cosmid vectors that contain two *cos* sites (e g , pcos2EMBL, Poustka et al 1984), provided digestion is carried out with the appropriate restriction enzymes

1. Add 20 μg of c2RB DNA to 20 μl of 10 × *Sma*I restriction enzyme buffer (1 × *Sma*I buffer should contain 20 mM KCl), and adjust the reaction volume to 200 μl with H_2O Add 40 units of *Sma*I, and incubate the reaction for 3 hours at room temperature

2. Analyze 2 μl (0 2 μg) of the reaction by electrophoresis through a 0 7% agarose gel to assess the extent of digestion by the restriction enzyme Use as markers (1) undigested c2RB DNA and (2) c2RB DNA digested with an excess of *Bam*HI If the reaction is incomplete (as evidenced by the continued appearance of superhelical or nicked circular forms of the plasmid DNA), add additional *Sma*I (20 units) and continue incubation for a further 2 hours at room temperature

If desired, the linearized vector may be dephosphorylated by alkaline phosphatase before addition of the second restriction enzyme Add 2 μl of 0 1 M $ZnCl_2$ and 0 25 unit of calf intestinal alkaline phosphatase directly to the reaction, and incubate for a further 30 minutes at 37°C Add SDS and EDTA (pH 8 0) to final concentrations of 0 5% and 5 mM, respectively Add proteinase K to a final concentration of 50 μg/ml Incubate the reaction for 30 minutes at 56°C Extract the solution once with phenol·chloroform and once with chloroform alone, and proceed as described in step 5, page 3 28. Redissolve the DNA in 180 μl of 10 mM Tris Cl (pH 7 6), and add 20 μl of the 10 × buffer appropriate for the second restriction enzyme

Dephosphorylation of the *Sma*I site is a further safeguard to prevent the formation of concatemers of vector DNA during ligation In most cases, however, this treatment is unnecessary

3. When digestion is complete, add NaCl to a final concentration of 150 mM and add 40 units of *Bam*HI. After incubating the reaction for 3 hours at 37°C, analyze an aliquot (5 μl; 0.5 μg) by electrophoresis through a 0.7% agarose gel. Only two bands (1.7 kb and 5.1 kb) should be visible; no linear plasmid DNA should remain. If digestion is incomplete, add additional *Bam*HI (20 units) and continue incubation for a further 2 hours at 37°C

   Eukaryotic DNA can also be cloned at the *Eco*RI, *Hin*dIII, or *Cla*I sites of c2RB If any of these sites are to be used, adjust the conditions of digestion with the second restriction enzyme accordingly

4. When digestion is complete, extract the solution once with phenol chloroform and once with chloroform alone. Precipitate the DNA with 2 volumes of ethanol at 0°C for 5 minutes Recover the DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge and redissolve it in 40 μl of 10 mM Tris Cl (pH 8 0).

5. Set up the ligation and packaging reactions as described on page 3 40 Note that the ligation reaction should contain ATP at a final concentration of 5 mM (Ferretti and Sgaramella 1981)

   If dephosphorylated vector is used, the concentration of ATP in the reaction can be reduced to the standard 0 5–1 mM

Amplification and Storage of Cosmid Libraries

Cosmids packaged in bacteriophage λ particles are stable for several weeks when stored in SM containing a few drops of chloroform If sufficient recombinant cosmids are obtained in the original packaging reactions, there may be no need to amplify the library prior to screening. Most investigators, however, are reluctant to commit their entire stock of recombinant cosmids to a single screening exercise In most cases, they prefer to amplify the library despite the risk of distortion caused by unequal growth of the cosmids

There are three ways to amplify and store cosmid libraries

1. Transduce the packaged cosmids into bacteria, grow the bacteria on nitrocellulose filters on Terrific Broth (TB) agar plates ($\sim 5 \times 10^4$ colonies per 138-mm filter), and replicate the library onto additional filters for screening and storage at −70°C (Hanahan and Meselson 1980) This method is tedious, requires the preparation and storage of a large number of filters (as many as 30 filters per library), and is sometimes plagued by loss of the colonies that do not regrow from the master filter However, it has the advantage of minimizing distortion of the library, at no stage are mixed populations of bacteria containing different recombinant cosmids growing in competition with one another. It is therefore unlikely that severe imbalances will arise as a consequence of differential growth of the host bacteria
2. Transduce the packaged cosmids into bacteria, plate the bacteria onto selective plates, and scrape the resulting colonies from the plate into TB containing the appropriate antibiotic Grow the mixed population of bacteria for a few generations, and store the culture at −70°C, aliquots are thawed and plated as needed. This procedure is less time-consuming than the first and does not require storage and handling of a large number of filters. However, there is always the concern that some bacteria carrying recombinant cosmids may not grow as vigorously as others, resulting in over- or underrepresentation of certain sequences in the cosmid library
3. Transduce the packaged cosmids into bacteria, plate the bacteria onto selective plates, and scrape the resulting colonies into TB containing the appropriate antibiotic Grow the mixed population of bacteria for a limited number of generations, and then superinfect with a strain of bacteriophage λ that is recombination-deficient (*red*$^-$) and mutant in a lysis function (usually *S*am7) The cosmid resident in the superinfected bacterium becomes packaged in vivo and appears in the yield of bacteriophage particles, which can be stored without loss of viability for prolonged periods of time. This is the most complicated method of amplification, and it contains several steps (growth of bacteria, superinfection, and packaging) at which selection for or against individual recombinants might occur

None of these protocols is ideal. Either they are labor-intensive or they may lead to differential representation caused by slow growth of the bacterial host or poor packaging of the cosmid in vivo (see, e.g., Gibson et al. 1987b). In some cases, the method of amplification is dictated by how the library will ultimately be used. For example, screening a cosmid library by recombination (Poustka et al. 1984; Lindenmaier et al. 1985) requires the generation of bacteriophage particles that can transduce the cosmids into recombination-competent bacteria In this case, method 3 is the best choice For routine screening of cosmid libraries by hybridization, we recommend method 1 as the safest choice.

Replica Filters

In this method, bacterial colonies grown on nitrocellulose filters laid on agar plates containing the appropriate antibiotic are frozen in situ essentially as described by Hanahan and Meselson (1980) Approximately 5×10^4 colonies can be accommodated on a 138-mm filter; approximately 30 filters are therefore required to obtain a representative library of overlapping fragments of a mammalian genome Replicas are then prepared from the set of master filters for screening and propagation of the library

1. Prepare ahead of time
   a. TB agar plates containing the appropriate antibiotic.
   b. TB agar plates containing the appropriate antibiotic and 25% glycerol (freezing plates)
   c. Detergent-free nitrocellulose filters (Millipore HATF or equivalent) These are prepared as follows
      i Calculate the number of filters required·

Diameter of plate[a]	Diameter of filter	No. colonies/filter
90 mm	82 mm	$1\,5 \times 10^4$
150 mm	138 mm	$5\,0 \times 10^4$

[a] These are the sizes as specified by certain manufacturers The actual internal plate diameters are less (e g , a 90-mm plate has an effective diameter of 82 mm)

      ii Number the dry filters with a soft-lead pencil or an ultrathin ballpoint pen Float each filter separately on the surface of a tray of water until it is thoroughly wet, and then submerge it for several minutes When all of the filters have been treated in this way, remove them from the water one by one and sandwich each filter between two dry Whatman 3MM papers Wrap the stack of filters in aluminum foil, and sterilize them by autoclaving (30 minutes at 15 lb/sq. in on liquid cycle) Store the stack of sterile filters at room temperature in a sealed plastic bag until required
      iii. Pick up a numbered, sterile filter with sterile forceps and lay it, numbered side down, on the surface of a day-old TB agar plate When the filter is thoroughly wet, peel it from the plate and replace it, numbered side up, on the same plate

2. Dispense aliquots of the packaging mixture containing 10^4 packaged cosmids into tubes containing 200 μl of a fresh overnight culture of a *recA*$^-$ strain of *E. coli* (e g., ED8767 or DH1) grown in TB in the presence of 10 mM $MgCl_2$ and 0 2% maltose Incubate the cultures for 20 minutes at 37°C.

   Large amounts of packaging mixture can inhibit attachment of the bacteriophage particles to the bacteria and can kill cosmid-containing bacteria. Therefore, if the concentration of cosmids is low ($<10^4$ cosmids per milliliter of packaging mixture), carry out the infection with more cells in a larger volume (e.g , 10 ml of bacterial culture for every milliliter of packaging reaction). After step 3, recover the bacteria by centrifugation at 5000*g* for 10 minutes at 4°C. Resuspend the bacteria in 100 μl of TB for every milliliter of original bacterial culture, and continue as described in step 4

ED8767 and DH1 are less "leaky" *recA*$^-$ strains than HB101 and may therefore more effectively suppress recombination between repetitive sequences of cloned eukaryotic DNA

3. Add 0 5 ml of TB to each tube and continue incubating the cultures for a further 45 minutes at 37°C to allow expression of the ampicillin resistance gene.

4. Apply 0 5 ml of the infected cell culture to the center of a numbered filter (138 mm) on the surface of a TB agar plate containing the appropriate antibiotic. Using a sterile glass spreader, disperse the fluid evenly over the surface of the filter. Leave a border 2–3 mm wide at the edge of the filter free of bacteria After spreading the inoculum, incubate the plate (noninverted) with the lid ajar for a few minutes in a laminar flow hood to allow the inoculum to evaporate Then close the lid, invert the plate, and incubate at 37°C until small colonies (0 1–0 2 mm diameter) appear (about 8–10 hours)

Plates that are 2–3 days old will absorb the inoculum more efficiently than freshly made plates

5. If desired, replica filters may be prepared at this stage (see step 7, page 3.49) Otherwise, transfer the filters (colony side up) to TB agar plates containing the appropriate antibiotic and 25% glycerol Incubate the plates for 2 hours at 37°C

6. Seal the plates well with Parafilm, seal in a plastic bag, and store in an inverted position at −70°C. Replicas can be made after thawing the master plates at room temperature (still in an inverted position)

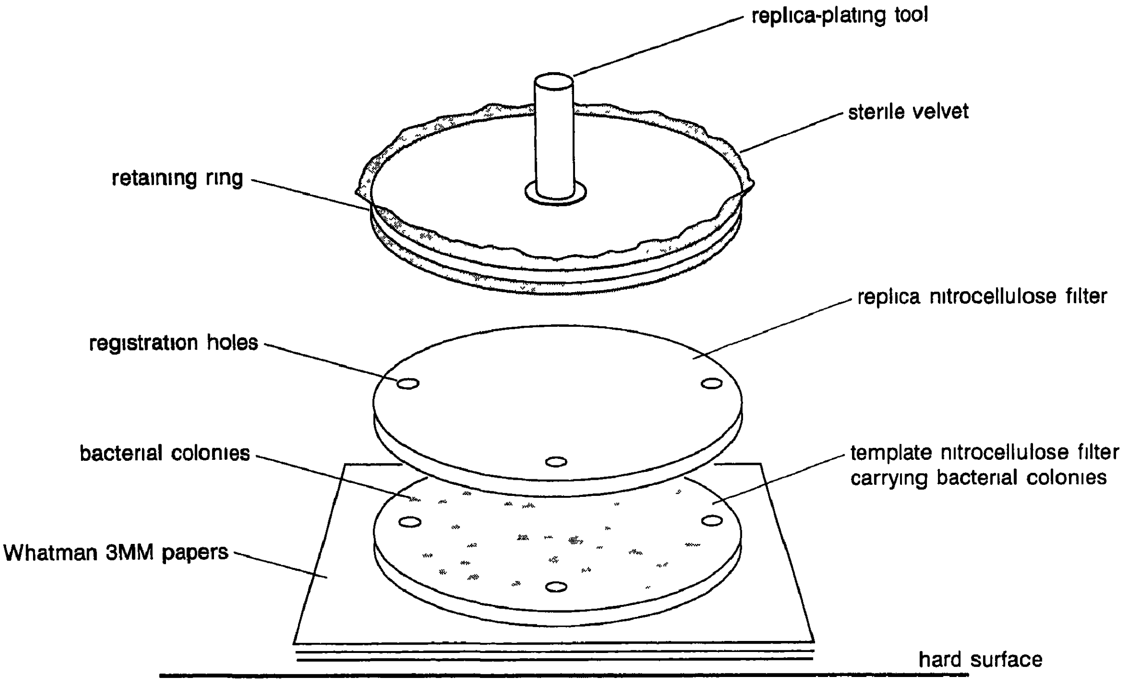


FIGURE 3.6
Use of a replica-plating tool to replicate bacterial colonies growing on nitrocellulose filters

7. Make replica filters as follows:

   a Prepare in advance a stack of sterile Whatman 3MM papers (one for each filter plus a few spares)

   b Peel the master nitrocellulose filter off the storage plate and lay it, colony side up, on a dampened pad of sterile 3MM paper.

   c Number a damp, sterile nitrocellulose filter and lay it on the master nitrocellulose filter. Take care to prevent air bubbles from becoming trapped between the two filters This is best done by bending the second filter slightly so that contact is first made at the center of the filters Be careful not to move the filters relative to each other once contact has been made. Try to arrange the two filters so that they do not overlap exactly; this makes it easier to separate the two filters

   d Press the two filters firmly together with a velvet replica-plating tool (Figure 3.6) or with a heavy glass plate

   e Orient the two filters by making a series of holes in the pair of filters with an 18-gauge needle

   f Peel apart the filters Lay the replica on a fresh TB agar plate containing the appropriate antibiotic, and incubate at 37°C until colonies appear (4–6 hours)

   g Place the master nitrocellulose filter on a fresh TB agar plate containing the appropriate antibiotic and 25% glycerol Incubate the plate for 1 hour at 37°C, and then freeze it as described in step 6, page 3.47

   Replica filters can be used to replicate again, stored at −70°C, or used for screening by in situ hybridization

   To avoid contamination, filters containing copies of valuable libraries are best handled in a laminar flow hood

Amplification of Cosmid Libraries in Liquid Culture

Growing cosmid libraries in liquid culture is simpler and more convenient than serial replication of bacterial colonies on nitrocellulose filters. However, because the bacteria are grown as a mixed population in liquid, there is a possibility that the composition of the library may change because of differences in the growth rates of bacteria containing different cosmids. In the following procedure, cosmid-containing bacteria are grown initially on plates. This allows accurate quantitation of the number of independent cosmid clones and also allows a crude estimate of the variability in the rate of growth of the individual bacterial colonies. Care is taken to limit subsequent growth in liquid culture to minimize the possibility of significant shifts in the distribution of clones within the amplified population.

1. Dispense aliquots of the packaging mixture containing 10^4 packaged cosmids into tubes containing 100 μl of a fresh overnight culture of a *recA*$^-$ strain of *E. coli* (e.g., ED8767 or DH1) grown in Terrific Broth (TB) in the presence of 10 mM $MgCl_2$ and 0.2% maltose. Incubate the cultures for 20 minutes at 37°C.

   Large amounts of packaging mixture can inhibit attachment of the bacteriophage particles to the bacteria. Therefore, if the concentration of packaged cosmids is low, carry out the infection with more cells in a larger volume. After step 2, recover the bacteria by centrifugation at 5000*g* for 10 minutes at 4°C. Resuspend the bacteria in 100 μl of TB for every 10^4 packaged cosmids, and continue as described in step 3.

   ED8767 and DH1 are less "leaky" *recA*$^-$ strains than HB101 and may therefore more effectively suppress recombination between repetitive sequences of cloned eukaryotic DNA.

2. Add 0.5 ml of TB to each infected culture containing 10^4 packaged cosmids. Continue incubation for a further 45 minutes at 37°C.

3. Apply 0.5 ml of the infected cell culture to the center of a TB agar plate (150 mm) containing the appropriate antibiotic. Using a sterile glass spreader, disperse the fluid evenly over the surface of the agar. Leave a border 2–3 mm wide at the edge of the agar free of bacteria. Incubate the plate at 37°C until colonies (0.2–0.3 mm diameter) appear (about 12–14 hours).

4. Estimate the number of colonies, and then, using a glass spreading rod, scrape the colonies from each 150-mm plate into approximately 10 ml of TB. Rinse each plate with an additional 5 ml of TB to ensure complete recovery of the bacteria. Pool the bacterial suspensions obtained from different plates.

5. Vortex the pooled, scraped, cell suspension to break up clumps of bacteria. Add sterile glycerol to a final concentration of 15%, mix well by vortexing, and dispense aliquots of the bacterial suspension into 50–100 sterile vials.

6. Store each aliquot at −70°C. The following day, remove an aliquot, thaw it rapidly at 37°C, and then titrate the number of viable cells. The titer of the library should remain constant for at least 1 year.

7. To screen the library, thaw an aliquot rapidly at 37°C and plate the appropriate number of bacteria on nitrocellulose filters or directly on TB agar plates containing the appropriate antibiotic.

Preparation of a Transducing Lysate of Packaged Cosmids

Because circular cosmid molecules carried in bacterial cells contain a *cos* site, they can be efficiently packaged into bacteriophage λ particles. When cosmid-containing bacteria are infected with bacteriophage λ (or when a resident prophage is induced), the *cos* site in the cosmids is cleaved by the bacteriophage λ *ter* function. The linearized cosmid DNA is then packaged into the heads of newly formed bacteriophage λ particles, which can be readily isolated and used to transduce the cosmid genome into other cells. The ability to rescue the cosmid DNA in the form of bacteriophage particles provides a convenient method to amplify cosmid libraries

Exactly how cosmid DNA is packaged into bacteriophage λ particles during the course of bacteriophage infection is not known However, the requirement for the bacteriophage λ *gam* gene product (the inhibitor of the host *recBC* nuclease) suggests that the cosmid may undergo rolling-circle replication during the infection process (Feiss et al. 1982). Other factors that affect the efficiency of the packaging process in vivo include

- *The genetic markers carried by the superinfecting bacteriophage* To reduce the level of generalized recombination that may lead to the instability of inserts cloned within cosmids, *red*$^-$ bacteriophages are used In addition, the superinfecting bacteriophage is usually defective in lysis (commonly *S*am7) for superinfection of cosmid-containing bacteria An alternative proced- is prolonged, leading to the accumulation of large numbers of mature bacteriophage particles in the infected cell
- *The structure of the cosmid* Lindenmaier et al. (1982) have shown that cosmid vectors with duplicated *cos* sites appear to be packaged with two to four times higher efficiency than those carrying a single *cos* site
- *The size of the cosmid DNA to be packaged* Cosmid libraries contain clones that range in size from 35 kb to 45 kb From everything we know about packaging, it would be unreasonable to think that cosmids with different sizes will be packaged in vivo with equal efficiency Consequently, packaging of cosmids is likely to distort still further the representation of sequences in the library

The protocol given below describes the use of λ3169 (*imm*434 *cIts b*2 *red*3 *S*am7) for superinfection of cosmid-containing bacteria. An alternative procedure is to transduce the cosmids initially packaged in vitro into a lysogenic strain of bacteria and then to induce the lysogen However, this approach is not commonly used because of the variability in the yield of recombinant cosmids in such lysogens

1. Dispense aliquots of the packaging mixture containing 10^5 packaged cosmids into tubes containing 1 ml of a fresh overnight culture of a *recA*$^-$ strain of *E coli* (e.g., ED8767 or DH1) grown in Terrific Broth (TB) in the presence of 10 mM $MgCl_2$ and 0.2% maltose. Incubate the cultures for 20 minutes at 37°C.

Large amounts of packaging mixture can inhibit attachment of the bacteriophage particles to the bacteria Therefore, if the concentration of packaged cosmids is low, carry out the infection with more cells in a larger volume After step 3, recover the bacteria by centrifugation at 5000*g* for 10 minutes at 4°C Resuspend the bacteria in 1 ml of TB for every 10^5 packaged cosmids and continue as described in step 4.

ED8767 and DH1 are less "leaky" *recA*$^-$ strains than HB101 and may therefore more effectively suppress recombination between repetitive sequences of cloned eukaryotic DNA

2. Add 3 ml of TB to each infected culture containing 10^5 packaged cosmids Continue incubation for a further 45 minutes at 37°C
3. Dilute the infected culture to an $OD_{600} = 0\ 05$ with TB containing the appropriate antibiotic, and grow the culture at 37°C with strong agitation until the $OD_{600} = 0.3$
4. Add approximately 1×10^{10} pfu of λ3169, and immediately raise the temperature of the culture to 42°C to prevent the formation of lysogens. This is best achieved by shaking the culture in a water bath set at 42°C.
5. After 30 minutes, transfer the culture to 37°C and continue incubation with vigorous agitation for 3 hours
6. Harvest the cells by centrifugation at 5000*g* for 10 minutes at 4°C, and resuspend the cells in 1/20 volume of SM Add a few drops of chloroform, and shake the bacterial suspension for 10 minutes at room temperature
7. Remove the bacterial debris by centrifugation at 5000*g* for 15 minutes at 4°C, and measure the titer of the transducing bacteriophage in the supernatant by infecting susceptible bacteria and determining the number of colonies on TB agar plates containing the appropriate antibiotic. Titers of 10^9 to 10^{10} transducing particles per milliliter of original infected culture should be expected.
8. Store the transducing particles in the same way a stock of bacteriophage λ is stored (see Chapter 2, page 2 68)

Note

The packaging of a single recombinant cosmid clone into transducing particles of bacteriophage λ is a convenient method to prepare cosmid DNA. Normally, the cosmid DNA is contaminated by sequences of the helper bacteriophage, which are also packaged into particles However, if the components for in vivo packaging are provided by strains of bacteriophage λ that carry defective *cos* sequences (Rosenberg et al. 1985), the cosmid DNA can be obtained in a form pure enough to allow the construction of restriction maps. The fact that the packaged cosmid DNA is linearized at the *cos* site is a significant help for this purpose (see Rackwitz et al 1984).

Problems Commonly Encountered When Using Cosmids

With the design of better vectors, two of the chief difficulties encountered in the construction of cosmid libraries—self-ligation of vector molecules and the insertion of more than one piece of foreign DNA per vector—have now been solved. Even so, construction of genomic DNA libraries in cosmids is not a routine matter and remains a more difficult task than constructing libraries in bacteriophage λ vectors Often, several attempts are required to obtain a library that is both large and representative

As in any complex project, the best advice is to check each reagent and construct ahead of time. Wherever possible, verify that a step in the protocol has worked before moving on to the next The problems that commonly arise are described below For some of them, the solutions are obvious, in other cases, however, difficulties cannot easily be resolved, since they stem from the biological properties of cosmids themselves as well as from those of the host bacteria.

1 The most frequent cause of failure is the quality of the genomic DNA used to construct the library Unless the length of the starting DNA exceeds 200 kb, only a small fraction of the 35–45-kb molecules obtained by partial digestion with a restriction enzyme will carry restriction sites at both ends Check the size of the starting DNA by electrophoresis through a 0 3% agarose gel or by pulsed-field gel electrophoresis (see Chapter 6) Do not attempt to construct a library unless the average size of the DNA exceeds 200 kb

2 Difficulties often arise in obtaining satisfactory partial digestion of high-molecular-weight eukaryotic DNA. Most commonly, this is caused by the presence of inhibitors in the DNA preparation When preparing high-molecular-weight DNA, take care to avoid material at the interface of the organic and aqueous phases If the problem persists, the DNA can be purified by equilibrium centrifugation in cesium chloride gradients (see Chapter 2)

3 Libraries constructed in cosmid vectors frequently contain clones that carry no inserts of foreign DNA The proportion of these clones varies according to the vector used and the care taken in its preparation Typically, 5% of the clones in libraries constructed in cosmids carrying a single *cos* site are empty (i.e , they contain no inserts) This proportion is much lower when vectors carrying two *cos* sites are used (Bates and Swift 1983), but it is considerably higher (20% or greater) in libraries constructed in charomid vectors (Saito and Stark 1986) This abnormally high background, whose cause is unknown, is a nuisance for two reasons: It increases the total number of cosmid-containing colonies that must be screened, and it poses problems during amplification of libraries, since the empty clones are likely to have a competitive advantage.

4. The copy number of different recombinant cosmids can vary widely, causing large differences in yield These differences become magnified every time a stock of transducing bacteriophage particles is prepared from a library The causes of this variability in yield are not totally understood but are likely to include interference with replication caused by (a) inverted repeat sequences that may block or retard the progress of the replication machinery and (b) transcription from sequences within the foreign DNA that fortuitously display promoter activity (Gibson et al 1987b).

5 Although the library may seem to be comprehensive, it may fail to yield the clone of interest Among the large number of factors that may account for this phenomenon are nonrandom distribution of restriction sites in the foreign DNA, restriction activity in the packaging extracts prepared from certain strains of *E coli* (see Rosenberg 1985), deletion of sequences by recombination, and selection against representation of the clone of interest during amplification of the library

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4

Single-stranded, Filamentous Bacteriophage Vectors

The filamentous bacteriophages specific to *Escherichia coli*, which include M13 (Hofschneider 1963), f1 (Loeb 1960; Zinder et al. 1963), and fd (Marvin and Hoffmann-Berling 1963), each contain a single-stranded closed circular molecule of DNA approximately 6400 nucleotides in length. When used as cloning vectors, these bacteriophages have the unique advantage of generating large quantities of DNA molecules that carry the sequence of one strand of the foreign DNA. Such single-stranded DNAs are the templates of choice for:

- DNA sequencing by the dideoxy chain-termination method
- Generating DNA probes for hybridization that are radiolabeled in only one strand
- Site-directed mutagenesis using synthetic oligonucleotides

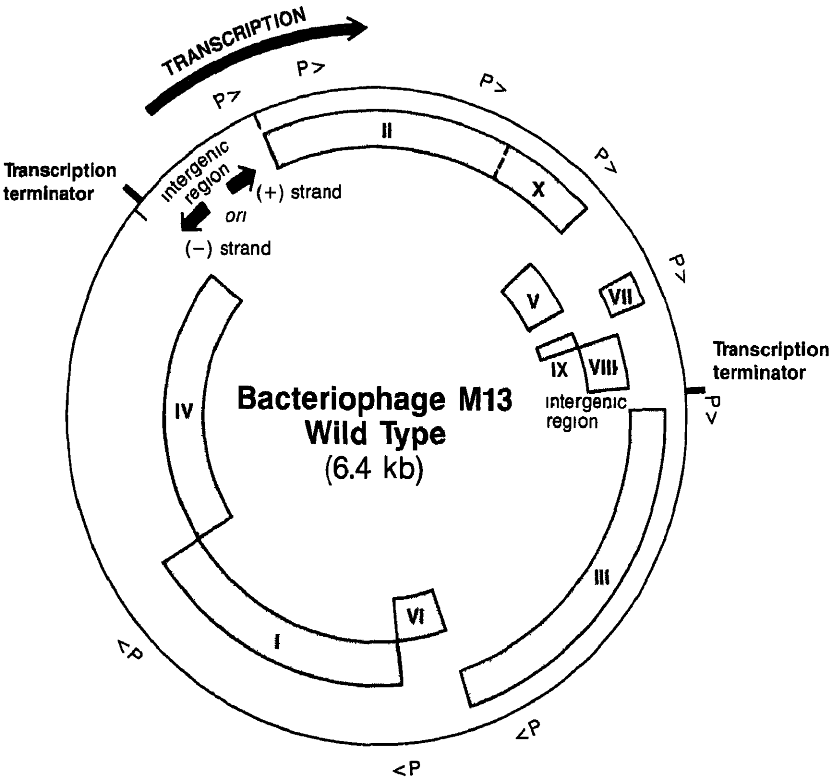


FIGURE 4.1
The genome of wild-type bacteriophage M13 is a single-stranded circular DNA 6407 nucleotides in length that is numbered in a 3′→5′ direction from the unique *Hpa*I site (van Wezenbeek et al. 1980). The figure shows the approximate locations of the genes (which are given Roman numerals), the positions of the major promoters (P>), and the terminators of transcription. By convention, the genome of the bacteriophage is called the (+) strand; it is synthesized in the direction gene II→gene IV. The coding (sense) strand of the viral genome is the (−) strand.

The Biology of Filamentous Bacteriophages

M13, f1, and fd are very closely related· Their genomes are organized identically, their particles are similar in size and shape, and their DNAs are highly homologous The genomes of these bacteriophages have been completely sequenced (fd, Beck et al 1978, M13, van Wezenbeek et al 1980; f1, Beck and Zink 1981) More than 98% of their nucleotide sequences are identical, the few differences are scattered around the genome, mostly in the third position of redundant codons (Beck and Zink 1981) M13, fd, and f1 actively complement and recombine with one another (Lyons and Zinder 1972) and may be regarded as identical for the purposes of the discussion that follows

The genetic map of bacteriophage M13 is shown in Figure 4 1 Over 90% of the viral genome codes for protein products, and most of the ten genes are separated by only a few nucleotides The only extended intergenic regions lie between genes VIII and III and between genes II and IV These regions contain the elements that regulate gene expression and viral DNA synthesis The restriction map of the DNA of wild-type bacteriophage M13 is presented in Figure 4 2

The product of gene VIII is the major structural protein of the bacteriophage particles, forming a tubular array of approximately 2700 morphological subunits surrounding the viral genome The resulting filamentous virus particles are only able to infect bacterial strains that express sex pili encoded by an F factor A few copies of a minor coat protein (the product of gene III), located at one end of the filamentous rod, are involved in the adsorption of the virus particle to the end of the bacterial pilus As the rod-shaped virus penetrates the pilus, its major coat protein is removed and the viral DNA, together with its attached gene III protein, is transferred to the body of the bacterium The infecting single-stranded DNA, designated the (+) strand, is then converted by cellular enzymes into a double-stranded circular form, called replicative form (RF) DNA Transcription of viral genes begins at any one of a series of promoters and proceeds unidirectionally to one of two terminators, which are located immediately downstream from genes VIII and IV This organization of promoters and terminators leads to gradients of transcription in which genes located closest to a termination site (e g , gene VIII) are transcribed much more frequently than genes further upstream (e g , gene II)

Amplification of the viral genome begins when the protein product of gene II introduces a nick at a specific site in the (+) strand of the parental RF DNA (Meyer et al 1979) *E coli* DNA polymerase I then adds nucleotides to the free 3′-hydroxyl terminus, progressively displacing the original (+) strand from the circular (−) strand template (Figure 4 3) After the replication fork has completed a full circle, the displaced (+) strand is cleaved by the gene II product to generate a unit-length viral genome that is then circularized (Horiuchi 1980) During the first 15–20 minutes of infection, these progeny (+) strands are converted by cellular enzymes to closed circular RF DNA molecules, which serve as templates for further rounds of transcription and synthesis of additional (+) strands. By the time that 100–200 copies of the RF DNA have accumulated in the infected cell, there is enough single-stranded DNA-binding protein (the product of gene V) to (1) repress translation of gene II mRNA (Model et al 1982; Yen and Webster 1982) and (2) bind to the newly synthesized (+) strands, preventing their further conversion to RF DNA (Mazur and Zinder 1975) The number of RF DNA molecules in the infected cell and the rate of production of progeny (+) strands are therefore both kept within moderate limits (Mazur and Model 1973, Lerner and Model 1981).

The morphogenesis of filamentous bacteriophage particles is extraordinary Unlike most other bacterial viruses, they do not assemble bacteriophage particles intracellularly. Instead, the gene V protein–DNA complexes move to the bacterial membrane, where the gene V product is stripped from the (+) strand and the viral genome becomes covered with capsid protein as it is extruded through the membrane of the infected cell (for reviews, see Marvin and Wachtel 1975, Webster and Cashman 1978) Because the viral genome is not inserted into a preformed structure, there is no strict limit to the size of the single-stranded DNA that can be packaged Rather, the length of the filamentous particle varies according to the amount of DNA it contains Particles longer than unit length and containing multiple copies of the viral genome are found in stocks of all filamentous viruses (Salivar et al 1967; Scott and Zinder 1967, Pratt et al 1969), and inserts of foreign DNA seven

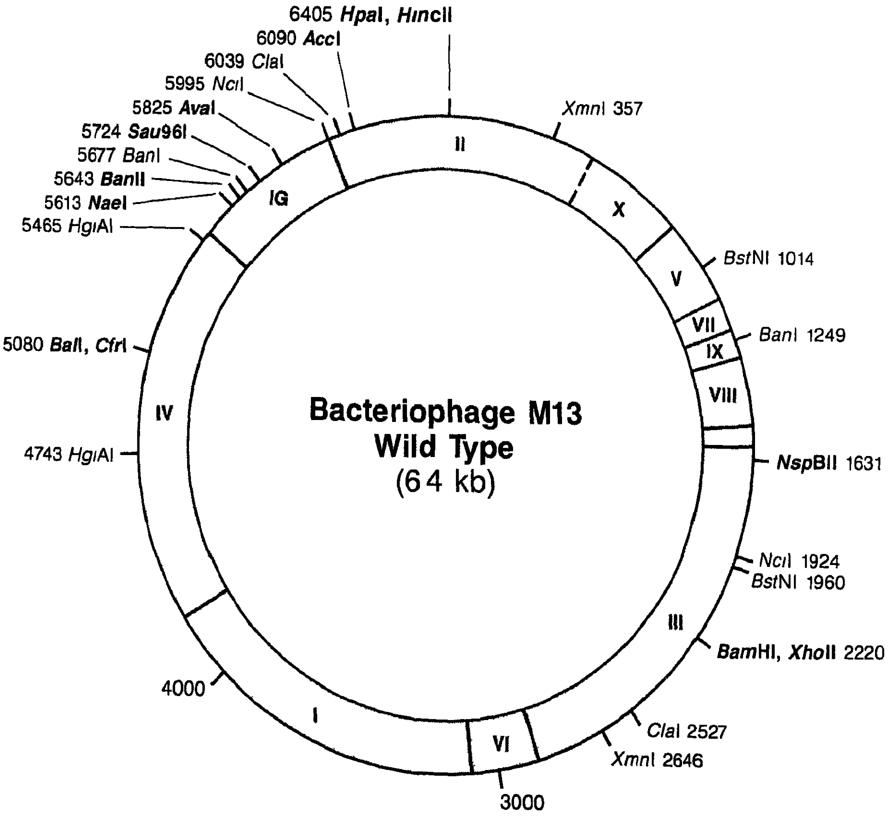


FIGURE 4.2

Restriction map of wild-type bacteriophage M13 (van Wezenbeek et al 1980) The figure shows the sites at which a number of restriction enzymes cleave the double-stranded replicative form of wild-type bacteriophage M13 DNA

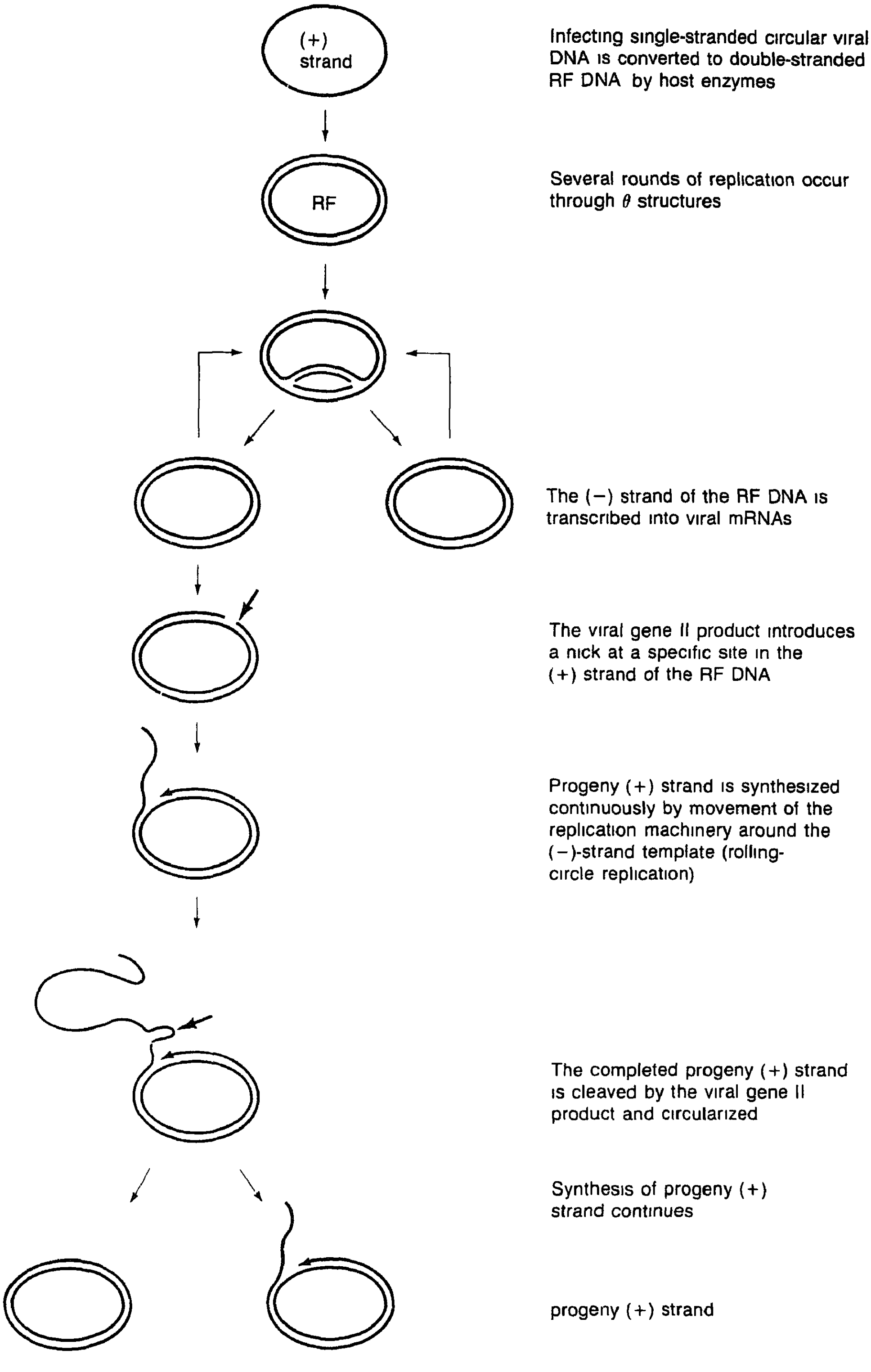

(+) strand
Infecting single-stranded circular viral DNA is converted to double-stranded RF DNA by host enzymes
RF
Several rounds of replication occur through θ structures
The (−) strand of the RF DNA is transcribed into viral mRNAs
The viral gene II product introduces a nick at a specific site in the (+) strand of the RF DNA
Progeny (+) strand is synthesized continuously by movement of the replication machinery around the (−)-strand template (rolling-circle replication)
The completed progeny (+) strand is cleaved by the viral gene II product and circularized
Synthesis of progeny (+) strand continues
progeny (+) strand

FIGURE 4.3

Replication of bacteriophage M13 DNA in infected bacteria The first four stages occur during the first 15–20 minutes of infection and result in the accumulation of 100–200 circular RF DNA molecules per cell. The subsequent production of single-stranded DNA is a continuous process that leads to morphogenesis of progeny particles times longer than the wild-type viral genome have been cloned and propagated in M13 (Messing 1981).

The replication of the filamentous bacteriophages occurs in harmony with that of the host bacterium, and the infected cells are therefore not lysed, but continue to grow (albeit at one half to three quarters of the normal rate) while producing several hundred virus particles per cell per generation (Marvin and Hohn 1969). This leads to the accumulation of vast numbers of particles in the medium; the titer of bacteriophage from a culture of infected bacteria frequently exceeds 10^{12} pfu per milliliter

Filamentous Bacteriophages as Vectors

All genes of filamentous bacteriophages are essential, and vectors must therefore carry a full complement of coding sequences. However, there is an ~500-nucleotide region, located between genes II and IV, into which segments of foreign DNA may be inserted. This noncoding region is not dispensable, since it contains a *cis*-acting signal for the packaging and orientation of the DNA within bacteriophage particles (Dotto et al. 1981; Webster et al. 1981), sites for the initiation and termination of synthesis of (+)- and (−)-strand DNA (see Beck and Zink 1981), and a signal for ρ-independent termination of transcription (see Konings and Schoenmakers 1978). Insertion of foreign DNA segments within this intergenic region can severely affect the replication of the bacteriophage genome (Dotto and Zinder 1984a). Fortunately, filamentous bacteriophages carrying foreign DNA sequences in the intergenic region accumulate mutations (in gene II or V) that partially compensate for the disruption of the *cis*-acting elements that control replication (Dotto and Zinder 1984b). All of the vectors in common use contain such mutations.

Because single-stranded DNA is a poor substrate for most restriction endonucleases and ligases, double-stranded segments of foreign DNA are inserted in vitro into the replicative form of the bacteriophage DNA. This double-stranded closed circular DNA can easily be purified from infected cells, manipulated in the same way as plasmid DNA, and then reintroduced into cells by standard transformation procedures. The double-stranded DNA then reenters the replication cycle, eventually generating progeny bacteriophage particles that contain only one of the two strands of the foreign DNA. The other strand of the DNA, the (−) strand, is never packaged.

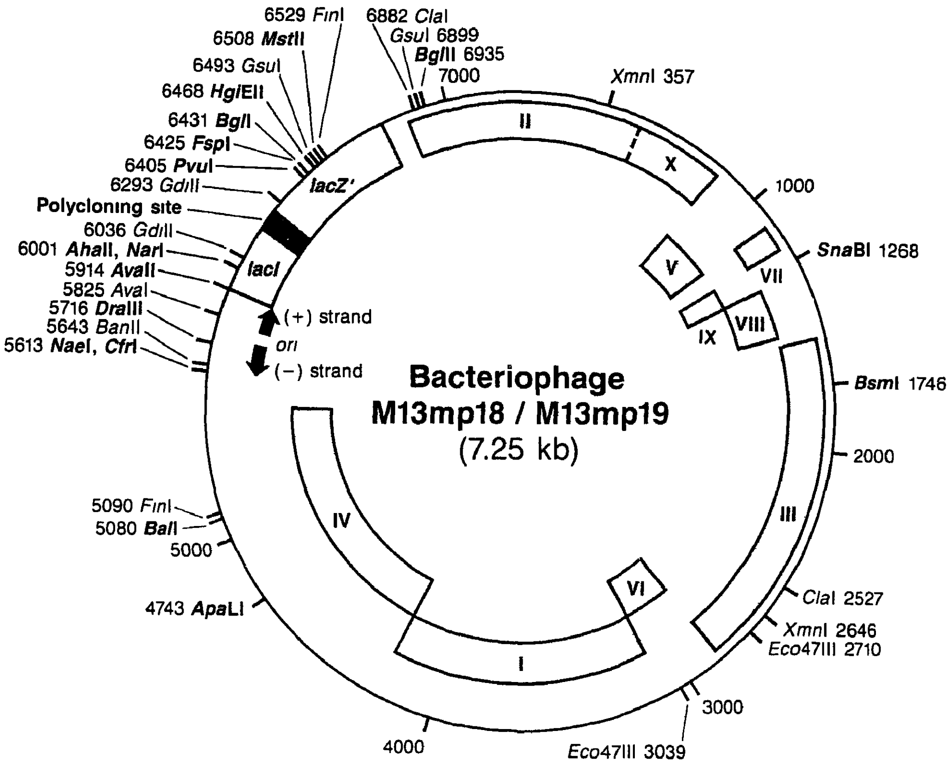
BACTERIOPHAGE M13 VECTORS

The region between genes II and IV has been used as the site for inserting foreign DNA into the mp series of bacteriophage M13 vectors developed by Messing and his co-workers (for review, see Messing 1983). The smaller region between genes VIII and III has served as the cloning site for another series of vectors (Barnes 1979, 1980) that are less frequently used.

Vectors of the mp series are all derived from a recombinant M13 bacteriophage (M13mp1) that carries a short segment of *E. coli* DNA in its major intergenic region (Messing et al. 1977). The presence of this segment, which contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lacZ*), has allowed the development of a simple color test to distinguish between vectors that carry a segment of foreign DNA and those that do not. The F′ plasmid of the host cell carries a defective β-galactosidase gene that codes for an enzymatically inactive polypeptide lacking amino acids 11–41. The amino-terminal fragment of the β-galactosidase protein produced in cells infected with an unsubstituted vector associates with this defective polypeptide to form an enzymatically active protein. This type of complementation in which deletion mutants of the operator-proximal segment of the *lacZ* gene are complemented by β-galactosidase-negative mutants that have their operator-proximal regions intact is called α-complementation (Ullmann et al 1967) Unsubstituted vectors plated on hosts carrying the appropriate F′ episome will therefore form blue plaques when the medium contains IPTG (isopropylthio-β-D-galactoside, a gratuitous inducer of β-galactosidase) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, a chromogenic substrate) Insertion of foreign DNA into the *lacZ* region of M13mp1 usually eliminates α-complementation and gives rise to recombinants that form pale blue or colorless plaques (Gronenborn and Messing 1978) The development of this simple test to score for recombinants led to other improvements (e g , the insertion of polycloning sites [see below]) that have made cloning in filamentous bacteriophages a routine event A number of cloning vectors have been described that carry other selectable markers (antibiotic resistance, *hisD*$^+$) that may be destroyed by insertion of a foreign DNA sequence (see, e g , Barnes 1979, Herrmann et al. 1980; Zacher et al. 1980) Unfortunately, these systems require the establishment of colonies of infected cells that must be screened by replica plating—a much slower and more tedious process than visual examination of plaques. Consequently, virtually all of the filamentous bacteriophage vectors in routine use are direct descendants of M13mp1

The segment of *lac* DNA carried in the intergenic region of M13mp1 contains very few useful cloning sites However, during the late 1970s and early 1980s, Messing and his colleagues used both chemical mutagenesis (Gronenborn and Messing 1978; Messing et al 1981) and site-directed mutagenesis (Messing et al 1981, Messing and Vieira 1982, Norrander et al 1983; Yanisch-Perron et al 1985) to build a useful series of densely packed restriction sites into the *lacZ* sequences of M13mp1 The presence of this polycloning site has little effect on the ability of the β-galactosidase peptide to carry out α-complementation However, insertions of additional DNA into the site generally destroy α-complementation and create recombinant bacteriophages that produce colorless plaques when grown in the presence of IPTG and X-gal

The cloning regions of vectors of the mp series that have been in common use during recent years are shown in Figure 4 5 (see pages 4 10–4 11) These vectors differ in the number and variety of restriction sites that can accept DNA fragments Currently, virtually all cloning is carried out with M13mp18 and M13mp19, which contain cloning regions with 13 different cleavage sites that can accept DNA fragments produced by digestion with many different restriction enzymes (Figure 4 4) The complete sequence of the DNA of M13mp18 and M13mp19 has been determined (Yanisch-Perron et al 1985) The two vectors differ only in the orientation of the nonsymmetrical polycloning region within the *lacZ* region After cleavage of the RF DNAs with two different restriction enzymes, M13mp18 and M13mp19 cannot recircularize easily unless a double-stranded foreign DNA fragment with compatible termini is present in the ligation mixture This fragment will be inserted in opposite orientations in the two vectors. Thus, one of the two strands of the fragment will be attached to the (+) strand of M13mp18, and the complementary strand will be attached to the (+) strand of M13mp19. Therefore, the progeny of the M13mp18 recombinant will contain one strand of the foreign DNA, and the progeny of the M13mp19 recombinant will contain the complementary sequence Consequently, by using M13mp18 and M13mp19 as dual vectors, it is possible to use a single primer ("universal primer") to determine the sequence of nucleotides on opposite strands from each end of the inserted DNA and to generate probes that are complementary to only one strand or the other of the foreign DNA



Polycloning Sites

M13mp18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC
					*Eco*RI		*Sac*I		*Kpn*I	*Sma*I *Xma*I		*Bam*HI		*Xba*I		*Sal*I *Acc*I *Hinc*II		*Pst*I		*Sph*I		*Hind*III				

M13mp19

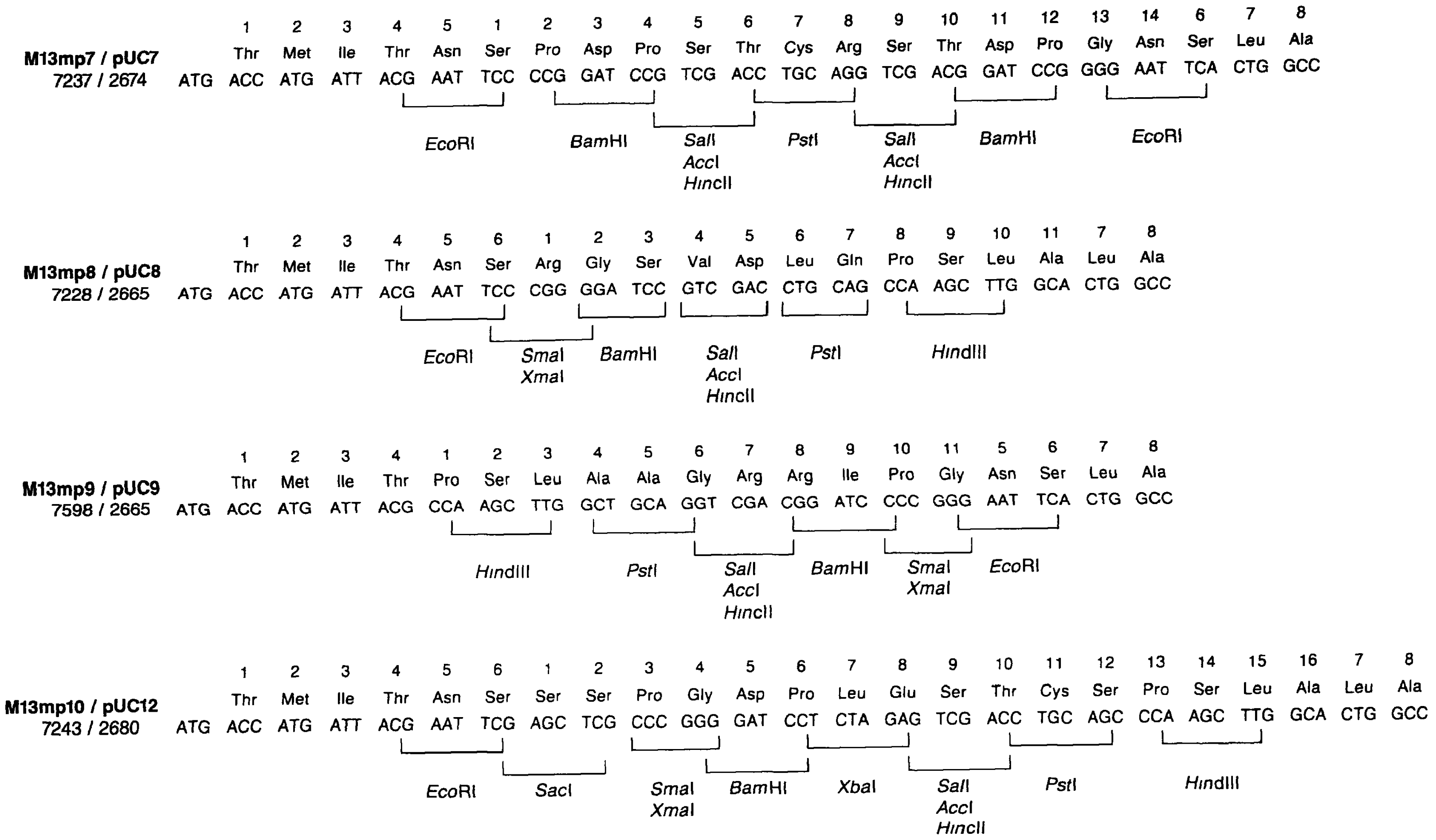
	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC
						*Hind*III		*Sph*I		*Pst*I		*Sal*I *Acc*I *Hinc*II		*Xba*I		*Bam*HI	*Sma*I *Xma*I		*Kpn*I		*Sac*I		*Eco*RI			

In M13mp18 the *Eco*RI site lies immediately downstream from P_{lac}

In M13mp19, the *Hind*III site lies immediately downstream from P_{lac}

FIGURE 4.4

Restriction map of bacteriophage M13 vectors M13mp18 and M13mp19 (Messing 1983, Norrander et al 1983, Yanisch-Perron et al 1985) The figure shows the sites at which a number of restriction enzymes cleave the double-stranded replicative forms of M13mp18 and M13mp19 The vectors differ from one another only in the orientation of the polycloning site


M13mp7 / pUC7
7237 / 2674
1 2 3 4 5 1 2 3 4 5 6 7 8 9 10 11 12 13 14 6 7 8
Thr Met Ile Thr Asn Ser Pro Asp Pro Ser Thr Cys Arg Ser Thr Asp Pro Gly Asn Ser Leu Ala
ATG ACC ATG ATT ACG AAT TCC CCG GAT CCG TCG ACC TGC AGG TCG ACG GAT CCG GGG AAT TCA CTG GCC
EcoRI
BamHI
SalI
AccI
HincII
PstI
SalI
AccI
HincII
BamHI
EcoRI
M13mp8 / pUC8
7228 / 2665
1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10 11 7 8
Thr Met Ile Thr Asn Ser Arg Gly Ser Val Asp Leu Gln Pro Ser Leu Ala Leu Ala
ATG ACC ATG ATT ACG AAT TCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TTG GCA CTG GCC
EcoRI
SmaI
XmaI
BamHI
SalI
AccI
HincII
PstI
HindIII
M13mp9 / pUC9
7598 / 2665
1 2 3 4 1 2 3 4 5 6 7 8 9 10 11 5 6 7 8
Thr Met Ile Thr Pro Ser Leu Ala Ala Gly Arg Arg Ile Pro Gly Asn Ser Leu Ala
ATG ACC ATG ATT ACG CCA AGC TTG GCT GCA GGT CGA CGG ATC CCC GGG AAT TCA CTG GCC
HindIII
PstI
SalI
AccI
HincII
BamHI
SmaI
XmaI
EcoRI
M13mp10 / pUC12
7243 / 2680
1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 7 8
Thr Met Ile Thr Asn Ser Ser Ser Pro Gly Asp Pro Leu Glu Ser Thr Cys Ser Pro Ser Leu Ala Leu Ala
ATG ACC ATG ATT ACG AAT TCG AGC TCG CCC GGG GAT CCT CTA GAG TCG ACC TGC AGC CCA AGC TTG GCA CTG GCC
EcoRI
SacI
SmaI
XmaI
BamHI
XbaI
SalI
AccI
HincII
PstI
HindIII

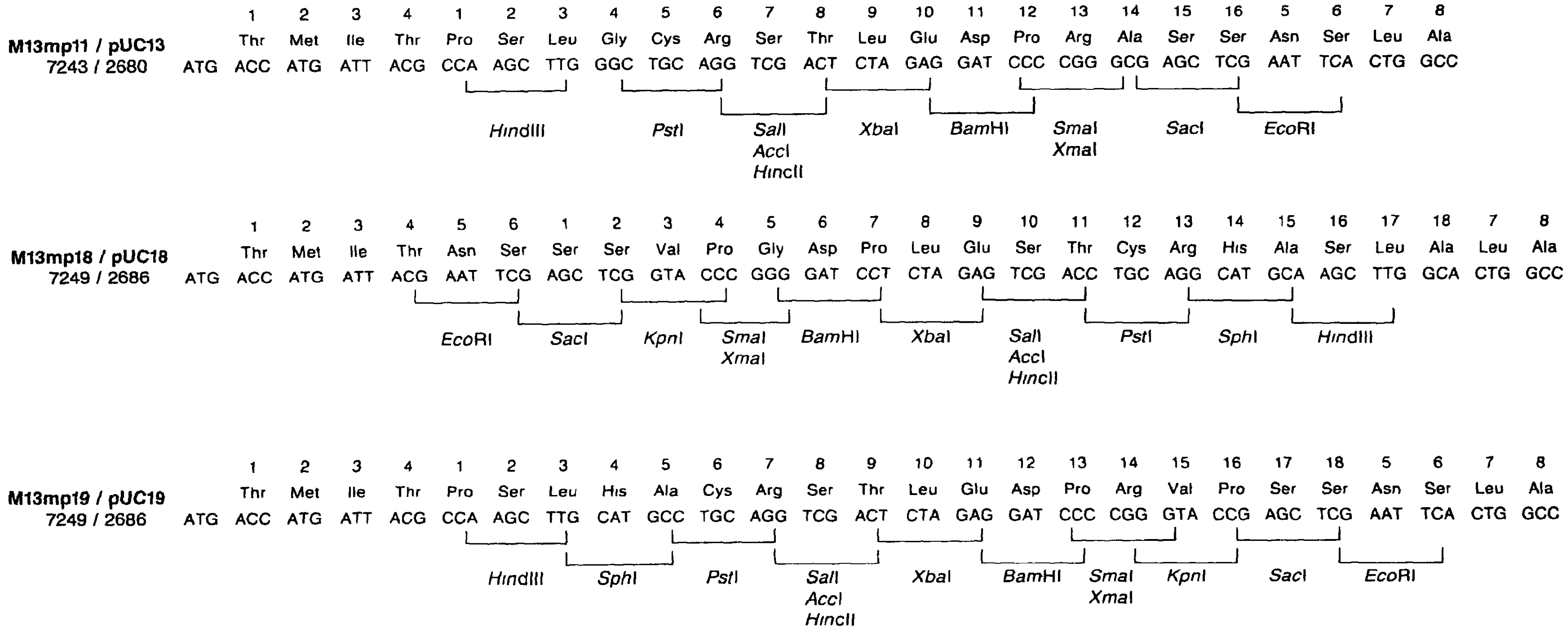

M13mp11 / pUC13
7243 / 2680
1 2 3 4 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 5 6 7 8
Thr Met Ile Thr Pro Ser Leu Gly Cys Arg Ser Thr Leu Glu Asp Pro Arg Ala Ser Ser Asn Ser Leu Ala
ATG ACC ATG ATT ACG CCA AGC TTG GGC TGC AGG TCG ACT CTA GAG GAT CCC CGG GCG AGC TCG AAT TCA CTG GCC
HindIII
PstI
SalI
AccI
HincII
XbaI
BamHI
SmaI
XmaI
SacI
EcoRI
M13mp18 / pUC18
7249 / 2686
1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 7 8
Thr Met Ile Thr Asn Ser Ser Ser Val Pro Gly Asp Pro Leu Glu Ser Thr Cys Arg His Ala Ser Leu Ala Leu Ala
ATG ACC ATG ATT ACG AAT TCG AGC TCG GTA CCC GGG GAT CCT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG GCA CTG GCC
EcoRI
SacI
KpnI
SmaI
XmaI
BamHI
XbaI
SalI
AccI
HincII
PstI
SphI
HindIII
M13mp19 / pUC19
7249 / 2686
1 2 3 4 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 5 6 7 8
Thr Met Ile Thr Pro Ser Leu His Ala Cys Arg Ser Thr Leu Glu Asp Pro Arg Val Pro Ser Ser Asn Ser Leu Ala
ATG ACC ATG ATT ACG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT TCA CTG GCC
HindIII
SphI
PstI
SalI
AccI
HincII
XbaI
BamHI
SmaI
XmaI
KpnI
SacI
EcoRI

FIGURE 4.5
Polycloning-site regions in the M13mp and pUC series of vectors The figure shows the positions of restriction sites in the polycloning-site regions of commonly used bacteriophage M13 vectors and their corresponding pUC plasmids The numbers beneath the names of the vectors are their approximate sizes (in nucleotides) Because the nucleotide sequences of most of these vectors have not been determined in their entirety, the sizes given are the best estimates that can be derived from information available in the literature (Yanisch-Perron et al 1985 and references therein)

The *lacZ* fragments used in the construction of the mp series of bacteriophage M13 vectors have also been inserted into a deleted version of pBR322, creating a family of plasmid vectors (pUC vectors) that contain a variety of commonly used cloning sites These vectors, which are described in detail in Chapter 1, are especially useful because they can participate in α-complementation and carry the same constellation of restriction sites in the polycloning site as the analogous vectors of the mp series (Figure 4 5) Thus, fragments of foreign DNA can be moved between M13 mp vectors and pUC vectors with great ease.

BACTERIAL HOSTS FOR BACTERIOPHAGE M13 VECTORS

Because M13 enters the host cell through sex pili encoded by an F factor, only male bacteria are used to propagate the virus[1] Bacterial strains carrying F′ episomes and a number of genetic markers useful in work with M13 vectors have been constructed by Messing and his co-workers (Yanisch-Perron et al 1985) The most important of these markers are

- *lacZ*ΔM15: A deletion mutant lacking the sequences of the *lacZ* gene coding for the amino-terminal portion of β-galactosidase (Beckwith 1964) The peptide expressed by ΔM15 can take part in α-complementation (Ullmann and Perrin 1970) Many hosts for bacteriophage M13 vectors carry this deleted version of the *lacZ* gene on an F′ episome

- Δ(*lac-proAB*). A deletion of the chromosomal segment spanning the *lac* operon and neighboring genes coding for enzymes involved in proline biosynthesis Bacterial hosts carrying this marker are unable to use lactose as a carbon source and require proline for growth

- *lacI*^{q} A mutant of the *lacI* gene that synthesizes approximately tenfold more repressor than wild type (Muller-Hill et al 1968) This overproduction of repressor suppresses still further the already low level of transcription of the *lac* operon in the absence of inducer Thus, synthesis of potentially toxic proteins is minimized when foreign coding sequences are placed under the control of the *lacZ* promoter in cells carrying *lacI*^{q} In most of the strains used in cloning with M13 vectors, *lacI*^{q} is carried with *lacZ*ΔM15 (see above) on an F′ episome

- *proAB*. The region of the bacterial chromosome coding for enzymes involved in proline biosynthesis. The *proAB* genes are often carried on an F′ episome, which can then complement proline prototrophy in a host with a (*lac-proAB*) deletion In this way, the maintenance of the F′ episome in the host can be guaranteed

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[1] Infections can be established in female cells if the bacteriophage DNA is introduced by transfection. However, the progeny particles produced by the transfected cells are unable to infect other cells in the culture, and the yields of virus are consequently very low This is therefore not recommended as a standard method for propagating M13 vectors and their recombinants

- *traD*36. A mutation that suppresses conjugal transfer of F factors (Achtman et al 1971). This marker was required by the NIH guidelines that were extant several years ago The guidelines are no longer in force, but the marker lingers on in several of the bacterial strains dating from that era

- *hsdR*17 and *hsdR*4: Mutations that lead to loss of restriction, but not modification, by the type I restriction/modification system of *E coli* strain K (for review, see Yuan 1981) Unmodified DNA cloned directly into M13 vectors and propagated in bacterial strains carrying this mutation will be modified and therefore protected against restriction if the vector is subsequently introduced into an *hsd*$^+$ strain of *E coli* K

- *recA*1 The *recA* gene of *E coli* codes for a DNA-dependent ATPase, 352 amino acids in length, that is essential for genetic recombination in *E coli* (for review, see Radding 1982) Strains that carry the *recA*1 mutation are defective in recombination and therefore have two advantages First, plasmids propagated in these strains remain as monomers and do not form multimeric circles (Bedbrook and Ausubel 1976) Second, segments of foreign DNA propagated in M13 vectors may experience fewer deletions in *recA*$^-$ strains (Yanisch-Perron et al 1985)

- *supE* An amber suppressor that inserts glutamine at UAG codons At one time, the NIH guidelines required bacteriophage M13 vectors to carry an amber mutation This requirement has long since been dropped, and most vectors in common use today do not carry such mutations However, many of the strains of *E coli* that are used as hosts were developed when the guidelines were still in force These strains and many of their derivatives carry a suppressor that allows the growth of vectors carrying certain amber mutations

Descriptions of strains of *E coli* commonly used in cloning in bacteriophage M13 vectors are given in Table 4 1

TABLE 4.1 Bacterial Strains Used to Propagate Bacteriophage M13 Vectors

Phenotype	Relevant genotype	Strain	Uses	Reference
	supE Δ(*lac-proAB*) F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	JM101^a	Permissive for vectors carrying amber mutations	Messing (1979)
r_k^-	*supE* Δ(*lac-proAB*) *hsdR*4 F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	JM105	Permissive for vectors carrying amber mutations Will modify but not restrict transfected DNA	Yanisch-Perron et al (1985)
r_k^-	*supE* Δ(*lac-proAB*) *hsdR*17 F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	JM107^b	Permissive for vectors carrying amber mutations Will modify but not restrict transfected DNA	Yanisch-Perron et al (1985)
r_k^- Rec$^-$	*supE* Δ(*lac-proAB*) *hsdR*17 *recA*1 F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	JM109$^{b\ c}$	Permissive for vectors carrying amber mutations Will modify but not restrict transfected DNA *recA*$^-$ strain	Yanisch-Perron et al (1985)
r_k^- Dam$^-$	*supE* Δ(*lac-proAB*) *hsdR*17 *dam* F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	JM110	Permissive for vectors carrying amber mutations Will modify but not restrict transfected DNA Will not modify *Bcl*I sites	Yanisch-Perron et al (1985)
r_k^- r_m^-	*supE* Δ(*lac-proAB*) *hsd*Δ5 F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	TG1	Permissive for vectors carrying amber mutations Will not modify or restrict transfected DNA	Gibson (1984)
r_k^- r_m^- Rec$^-$	*supE* Δ(*lac-proAB*) *hsd*Δ5 Δ(*srl-recA*)306 Tn*10*(*tet*^{r}) F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	TG2	Permissive for vectors carrying amber mutations Will not modify or restrict transfected DNA *recA*$^-$ strain	M Biggin (pers comm)
r_k^- Rec$^-$	*supE*$^+$ *lac*$^-$ *hsdR*17 *recA*1 F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	XL1-Blue	Permissive for vectors carrying amber mutations Will modify but not restrict transfected DNA *recA*$^-$ strain	Bullock et al (1987)

	Δ(*lac-proAB*) F′ *traD*36 *proAB* *lacI*^{q} *lacZ*ΔM15	XS127	Used for growth of phagemids	Levinson et al (1984)
r_k^- Rec$^-$ Kan^r	*hsdR recA*1 F′ *kan*	XS101	Used for growth of phagemids Will modify but not restrict transfected DNA *recA* strain Carries episome conferring resistance to kanamycin	Levinson et al (1984)
	supE Δ(*lac-proAB*) F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	71/18	Used for growth of phagemids	Dente et al (1983)
r_k^-	*supE* Δ(*lac-proAB*) *hsdR*4 F′ *traD*36 *proAB* *lacI*^{q} *lacZ*ΔM15	KK2186	Permissive for vectors carrying amber mutations Will modify but not restrict transfected DNA	Zagursky and Berman (1984)
Rec$^-$	Δ(*lac-proAB*) Δ(*srl-recA*)306 Tn*10*(*tet*^{r}) (φ80 *lacZ*ΔM15) F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	MV1184[d]	Used to propagate pUC118/pUC119 Used to obtain single-stranded copies of phagemids *recA* strain	Vieira and Messing (1987)

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[a] JM103 (Messing et al 1981) is a restrictionless derivative of JM101 that has been used to propagate bacteriophage M13 recombinants However, some cultivars of JM103 have lost the *hsdR*4 mutation (Felton 1983) and are lysogenic for bacteriophage P1 (which codes for its own restriction/modification system) JM103 is therefore no longer recommended as a host for bacteriophage M13 vectors Strain KK2186 (Zagursky and Berman 1984) is genetically identical to JM103 except that it is nonlysogenic for bacteriophage P1

[b] JM106 and JM108 are identical to JM107 and JM109, respectively, except that they do not carry an F episome These strains will not support the growth of bacteriophage M13 but may be used to propagate plasmids However, JM106 and JM108 do not carry the *lacI*^{q} marker (normally present on the F episome) and are therefore unable effectively to suppress the synthesis of potentially toxic products encoded by foreign DNA sequences cloned into plasmids carrying the *lacZ* promoter

[c] JM108 and JM109 are defective for synthesis of bacterial cell walls and form mucoid colonies on minimal media This does not affect their ability to support the growth of bacteriophage M13

[d] The original strain of MV1184, constructed by M Volkert (pers comm), did not carry an F′ episome However, the strain of MV1184 distributed by the Messing laboratory clearly carries an F′ episome It is therefore advisable to check strains of MV1184 on their arrival in the laboratory for their ability to support the growth of male-specific bacteriophages

PROBLEMS COMMONLY ENCOUNTERED WHEN CLONING IN FILAMENTOUS BACTERIOPHAGE VECTORS

Two types of problems are commonly encountered when cloning in filamentous bacteriophage vectors:

1. *Deletion of part of the foreign DNA segment.* DNA cloned into the intergenic region of filamentous bacteriophages tends to be unstable. The larger the cloned segment, the greater the rate at which deletions occur. This problem can be minimized (although never completely eliminated) by taking care never to propagate the bacteriophage by serial growth of infected cells in liquid culture. Instead, stocks of recombinant bacteriophages stored at −70°C should be plated on an appropriate host, and bacteria from the center of a single, well-isolated plaque should be used to establish a small-scale culture. This culture should provide sufficient quantities of single-stranded DNA or double-stranded RF DNA for most purposes. The culture should be grown for the shortest possible time (usually 4–8 hours, depending on the vector and the strain of *E. coli*) and should not be used as a stock to seed further cultures. Recombinant bacteriophages carrying larger segments of foreign DNA almost always grow more slowly than those carrying smaller inserts. Deletion of part of the foreign DNA sequences therefore confers a selective advantage that is often strong enough to result in the elimination of bacteria synthesizing the original recombinant within a few serial passages.
2. *Insertion of the foreign DNA in only one orientation.* Quite frequently, a given vector will carry a specific segment of foreign DNA far more readily in only one of the two possible orientations. This phenomenon occurs because sequences on opposite strands of the foreign DNA interfere to different extents with the functioning of the vector's intergenic region. This problem can sometimes be avoided by inserting the foreign DNA at a different site within the polycloning site or by using a combination of restriction enzymes that will allow directional cloning (see Chapter 1, pages 1.53–1.56). We have found, however, that it is not advisable to force bacteriophage M13 vectors to propagate unwelcome sequences of foreign DNA. Often, the resulting recombinants are unstable and give rise to progeny that carry deleted or rearranged versions of the foreign sequences. Instead, we recommend propagating the foreign DNA in plasmid vectors that carry an origin of replication derived from a filamentous bacteriophage (phagemids).

PHAGEMIDS: PLASMIDS CONTAINING AN ORIGIN OF REPLICATION DERIVED FROM A FILAMENTOUS BACTERIOPHAGE

Several vectors have been developed that combine desirable features of both plasmids and filamentous bacteriophages In their simplest form, these vectors are plasmids with a ColE1 origin of replication and a selectable marker for antibiotic resistance that carry in addition a copy of the major intergenic region of a filamentous bacteriophage This region contains all of the sequences required in *cis* for initiation and termination of viral DNA synthesis (Dotto et al 1984) and for morphogenesis of bacteriophage particles (Dotto and Zinder 1983) Segments of foreign DNA cloned in these vectors can be propagated as plasmids in the conventional way When cells harboring these plasmids are infected with a suitable filamentous bacteriophage, the mode of replication of the plasmid changes under the influence of the gene II product coded by the incoming virus. This gene II protein interacts with the intergenic region carried in the plasmid and initiates rolling-circle replication to generate copies of one strand of the plasmid DNA These single-stranded copies of the plasmid DNA will be nicked, circularized, and eventually become packaged into progeny bacteriophage particles (Dotto et al 1981, Dente et al 1983, Levinson et al 1984, Zagursky and Berman 1984, Geider et al 1985, Mead et al 1985) The single-stranded DNA purified from these particles can be used as a template to determine the nucleotide sequence of one strand of the foreign DNA segment, for oligonucleotide-directed mutagenesis, and for the synthesis of strand-specific probes A number of phagemids have been developed using intergenic regions derived from various filamentous bacteriophages and inserted into different plasmids. These phagemids are described in Table 4 2

Phagemids have several attractive features

- They provide the stability and high yields of double-stranded DNA that are characteristic of conventional plasmids.
- They circumvent the tedious and time-consuming process of subcloning DNA fragments from plasmids to filamentous bacteriophage vectors.
- They are sufficiently small that segments of foreign DNA up to 10 kb in length can be obtained in single-stranded form

However, many phagemids suffer from a major disadvantage—the sometimes poor and generally irreproducible yields of single-stranded DNA that are obtained after infection with helper bacteriophages Conventional bacteriophage M13 vectors grow to titers of $>10^{12}$ pfu/ml of bacterial culture; each milliliter of culture yields 5–10 μg of single-stranded viral DNA However, the yield of particles from cells carrying recombinant phagemids that have been infected by helper viruses can be 10- to 100-fold lower than that from filamentous bacteriophage vectors carrying the same segment of foreign DNA This reduction in yield is affected by a number of factors, including:

- *The exact sequences of the intergenic region that are contained in the phagemid* Plasmids carrying the complete intergenic region inhibit production of progeny particles by interfering with the replication of the DNA of the helper bacteriophage (Cleary and Ray 1980, 1981; Dotto et al. 1981).

TABLE 4.2 Phagemids

Plasmid	Intergenic region	Helper virus	Host bacteria[a]	Reference
pEMBL (derived from pUC8)	fl	IR1, an interference-resistant variant of fl[b]	71/18	Dente et al (1983)
pBR322	M13	wild-type M13	KK2186	Zagursky and Berman (1984)
pRSA101 (derived from πVX[c])	M13	a variant of M13 resistant to interference by plasmids containing the M13 intergenic region	XS127, XS101	Levinson et al (1984)
pUC118/119 (derived from pUC18/19)	M13	M13KO7 carries a mutated version of gene II that works less well on its own intergenic region than on that cloned in pUC118/119	MV1184	Vieira and Messing (1987)
pBluescript	fl	M13KO7	XL1-Blue	Short et al (1988)

Other more complicated phagemid systems have been devised (see, e g , Geider et al 1985, Mead et al 1985, Peeters et al 1986)

[a] See Table 4 1 for descriptions of bacterial strains

[b] Enea and Zinder (1982)

[c] Seed (1983)

This problem can be alleviated by using phagemids that carry intergenic regions from which the segment responsible for interference has been deleted (Cleary and Ray 1981, Zagursky and Berman 1984) or mutants of the helper that are resistant to interference (see below) (Enea and Zinder 1982; Levinson et al 1984) The inclusion in the phagemid of sequences coding for the carboxy-terminal region of the gene IV protein appears to lead to a further improvement in the yield of particles containing single-stranded plasmid DNA (Levinson et al 1984) The reason for this improvement is not known

- *The site at which the intergenic region is inserted into the plasmid* Phagemids carrying the intergenic region in the *Hin*dIII site of plasmid pBR322 interfere severely with the growth of wild-type bacteriophage (Levinson et al 1984), whereas those carrying the intergenic fragment in an *Aha*III site do not (Zagursky and Berman 1984).
- *The nature of the superinfecting bacteriophage* Both wild-type filamentous bacteriophages (Zagursky and Berman 1984) and interference-resistant mutants of these bacteriophages (Dente et al 1983; Levinson et al 1984, Mead et al 1985) have been used as helpers Although extensive comparisons of the efficiencies of the two kinds of helper have not been published, it seems that interference-resistant mutants may be the best helpers for small phagemids (those carrying a fragment of foreign DNA <5 kb in size), and that wild-type bacteriophages may be the best helpers for large phagemids (those carrying a fragment of foreign DNA >10 kb in size) (Levinson et al 1984) Vieira and Messing (1987) have devised a

clever way to overcome interference and to increase the proportion of progeny particles that contain single-stranded phagemid sequences. Their system uses a phagemid that contains an intergenic region derived from wild-type M13 DNA that is efficiently recognized by the gene II product of the infecting bacteriophage. The genome of the infecting bacteriophage (M13KO7) carries an intergenic region (derived from the mp vectors) that is not recognized as efficiently by the gene II product because it carries an insertion of *lacZ* sequences. The genome of the helper bacteriophage also contains a plasmid origin of replication and a selectable marker (resistance to kanamycin). Thus, when cells carrying a phagemid are superinfected, the incoming single-stranded genome is efficiently converted to a double-stranded form in the usual way. However, subsequent amplification of the helper DNA can be driven by the plasmid origin of replication, and the requirement for the gene II product is therefore greatly reduced or eliminated. Because the gene II product synthesized by the superinfecting bacteriophage interacts preferentially with the wild-type intergenic region carried by the resident phagemid, the majority of the resulting progeny (+) strands are derived from the phagemid genome and not from the newly established RF DNA. Often, the yield of single-stranded DNA contains 50-fold more phagemid sequences than helper sequences.

- *The size and nature of the foreign DNA cloned in the phagemid.* The yield of single-stranded DNA can vary over a five- to tenfold range depending on the size of the foreign DNA fragment (in general, the larger the fragment, the poorer the yield). Furthermore, for reasons that are not understood, foreign DNAs of equivalent size can suppress the yield of single-stranded DNA to varying extents.

In summary, phagemids show great potential as dual-purpose vectors. Unfortunately, the yield of single-stranded DNA from some systems varies in an unpredictable way and is often too low to provide sufficient template for more than one or two dideoxy sequencing reactions. Recently, however, Vieira and Messing (1987) have designed a system that overcomes some of these problems. Their phagemids (pUC118 and pUC119) are less sensitive than others to the size of the foreign DNA sequences and retain the advantages of cloning in pUC plasmids. Furthermore, when cells carrying these phagemids are infected with a suitable helper virus (M13KO7), they generally produce satisfactory yields of progeny particles containing single-stranded copies of the plasmid DNA (1×10^{11} to 5×10^{11} particles/ml).

The major advantage of phagemids is their ability to generate adequate amounts of single-stranded copies of segments of foreign DNA that are too large to clone in conventional M13 vectors without fear of deletion. Using the pUC118 and pUC119 phagemids, it is often possible to obtain enough single-stranded DNA from a large-scale preparation to carry out a series of sequencing reactions primed by synthetic oligonucleotides complementary to different portions of the insert. In this strategy, the last reliable sequence obtained from a sequencing reaction primed by one oligonucleotide is used to design the synthetic oligonucleotide used to prime the next. It is therefore possible to use a single phagemid to determine the entire sequence of one strand of the insert without resorting to subcloning into conventional M13 vectors.

THE USES OF SINGLE-STRANDED DNA GENERATED BY RECOMBINANTS CONSTRUCTED IN PHAGEMID AND FILAMENTOUS BACTERIOPHAGE VECTORS

Because their genomes tend to be unstable, recombinant filamentous bacteriophages are not used for the routine cloning and propagation of segments of foreign DNA. Instead, they are used chiefly to provide single-stranded copies of fragments of DNA that have already been cloned in another type of vector (a plasmid or phagemid) Single-stranded DNA can be obtained in this way with such ease and in such high yield that filamentous bacteriophage vectors (and more recently phagemids) have now completely replaced older, physical methods to separate the strands of segments of DNA (e.g., electrophoresis through strand-separating gels and digestion with bacteriophage λ exonuclease). Single-stranded DNA produced by these vectors is used as a template in three main types of reactions. dideoxy chain-termination DNA sequencing (Sanger et al 1977), oligonucleotide-directed mutagenesis (Zoller and Smith 1983), and primed synthesis of hybridization probes that are radiolabeled in only one strand These applications are described in detail in later chapters The remainder of this chapter deals with methods to propagate and store filamentous bacteriophages, to isolate single- and double-stranded DNAs, and to detect foreign DNA sequences cloned in filamentous bacteriophage vectors

Propagation of Bacteriophage M13 and Preparation of DNA

PLAQUE PURIFICATION OF BACTERIOPHAGE M13

Preparation of Plating Bacteria

1. Streak a master culture of a bacterial strain carrying an F′ episome (e.g., JM107 or JM109) onto a minimal (M9) agar plate. Incubate for 24–36 hours at 37°C

   The genes coding for enzymes involved in proline biosynthesis have been deleted from the chromosomes of these strains of bacteria Thus, only those bacteria that carry the F′ plasmid (F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) will be able to form colonies on media lacking in proline Bacteria grow much more slowly on minimal media than on rich media and do not survive prolonged periods of storage at 4°C Master stocks of bacteria carrying F′ episomes should therefore be stored at −70°C in LB medium containing 15% glycerol (see Appendix A)

2. Inoculate 50 ml of LB medium, in a 250-ml flask, with a single, well-isolated colony picked from the minimal agar plate Agitate the liquid culture for 6–8 hours at 37°C in a rotary shaker

   Stationary-phase cells tend to lose F′ episomes The plating culture should therefore be chilled to 0°C and placed in storage at 4°C before it reaches saturation Plating bacteria can be stored in this fashion for periods up to 1 week

Plating Bacteriophage M13

A plaque of bacteriophage M13 derives from infection of a single bacterium by a single virus particle. The progeny particles infect neighboring bacteria, which in turn release another generation of daughter virus particles. If the bacteria are growing in semisolid medium (containing, e.g., agar or agarose), the diffusion of the progeny particles is limited. Filamentous bacteriophages such as M13 do not kill the cells they infect, but they do slow their growth approximately twofold. Under these circumstances, a plaque forms as an expanding zone of slowly growing bacteria that eventually becomes visible to the naked eye against a background of more turbid bacterial growth within the agar or agarose overlay.

1. Prepare a series of labeled, sterile tubes (13 mm × 100 mm) containing 3 ml of melted top agar (made up in LB medium). Store the labeled tubes in a heating block at 47°C.

2. Prepare tenfold serial dilutions of the bacteriophage stock in LB medium. Dispense 10 μl or 100 μl of each dilution to be assayed into each of two labeled tubes (13 mm × 100 mm).

   A bacteriophage M13 plaque contains between 10^6 and 10^8 pfu. Medium from a liquid culture of bacteria infected with bacteriophage M13 contains between 10^{10} and 10^{12} pfu/ml.

3. Add 100 μl of plating bacteria (see page 4.21) to each tube. Mix by shaking or vortexing gently.

   Unlike bacteriophage λ, M13 adsorbs rapidly to bacteria, and there is no need to incubate the plating bacteria with the bacteriophage suspension before adding top agar.

4. Add 40 μl of a solution of X-gal (20 mg/ml in dimethylformamide) and 4 μl of a solution of IPTG (200 mg/ml) to one of the tubes containing top agar. Immediately pour the contents of this tube into one of the tubes containing plating bacteria and diluted bacteriophages. Mix the contents by gentle vortexing for 3 seconds, and then pour the mixture onto a labeled plate containing 30–35 ml of hardened LB agar medium, equilibrated to room temperature. Swirl the plate gently to ensure an even distribution of bacteria and top agar. Work quickly so that the top agar spreads over the entire surface of the agar before it sets.

   Prepare the X-gal solution by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution. Use a glass or polypropylene tube. Wrap the tube containing the solution in aluminum foil to prevent damage by light and store at −20°C. It is not necessary to sterilize X-gal solutions by filtration.

   Prepare the IPTG solution by dissolving 2 g of IPTG in 8 ml of H_2O. Adjust the volume of the solution to 10 ml and sterilize by filtration through a 0.22-micron disposable filter. Dispense the solution into 1-ml aliquots and store at −20°C.

5. Close the plates and allow the top agar to harden for 5 minutes at room temperature. Invert the plates and incubate at 37°C. Pale blue plaques will begin to appear after 4 hours, and the color will gradually intensify as

the plaques enlarge The plaques will be fully developed after 8–12 hours, and the blue color will intensify further if the plates are stored for a few hours at 4°C

Notes

i Colorless plaques (formed by bacteriophages that are unable to accomplish α-complementation) first appear after about 4–5 hours at 37°C and are best visualized after 8 hours of incubation

ii The color of the plaques is a useful, but not infallible, guide. Although nonrecombinant bacteriophages always give rise to blue plaques, not all blue plaques are necessarily formed by nonrecombinant bacteriophages. For example, recombinant bacteriophages carrying very small segments of foreign DNA that do not alter the reading frame of the *lacZ* sequences will code for fusion polypeptides that may still be partially competent for α-complementation These recombinants usually give rise to pale blue plaques, although in this case the color does not develop until the plates have been incubated for at least 6 hours Furthermore, at least one case has been reported in which a recombinant bacteriophage synthesizes a new α-donor whose first few amino acids are coded by the 3′ terminus of the foreign DNA and the remainder are from the original α-donor of the vector (Close et al 1983) This recombinant generates blue plaques that are indistinguishable from those formed by unsubstituted vector

iii Minimal (M9) agar plates can be used for plaque formation by bacteriophage M13 These have the advantage of allowing only bacteria that retain the F′ plasmid to grow Thus, infected cultures established from a plaque formed on minimal medium are unlikely to contain a significant number of female cells In contrast, plaques formed on richer medium (2 × YT or LB) may contain a proportion of auxotrophic female cells that are resistant to infection by bacteriophage M13. Fortunately, the spontaneous loss of F′ plasmids from bacteria grown for a limited period in rich medium is rarely high enough to cause problems Because plaques form more rapidly on richer medium than on minimal medium, we recommend that YT or LB medium be used for routine plating of bacteriophage M13

Picking Bacteriophage M13 Plaques

1. Dispense 1 ml of LB medium into a sterile tube (13 mm × 100 mm)

2. Touch the surface of the chosen plaque with the end of a sterile, 8-cm-long wooden stick (sometimes called applicator or orange stick). Immediately immerse the end of the stick in the LB medium and shake it vigorously to dislodge small fragments of agar (or agarose) Applicator sticks can be autoclaved and reused many times.

   Alternatively, plaques can be picked by either of the following methods

   i Attach a bulb to a sterile, disposable pasteur or capillary pipette and stab through the plaque into the underlying agar Then expel the plug of agar from the pipette into 1 ml of LB medium

   ii Touch the surface of the plaque with a sterile toothpick, and then drop the toothpick into a tube containing 1 ml of LB medium This method violates the rules of sterile technique, since the toothpick has been held by a nonsterile hand However, in practice, few problems arise, perhaps because the "dirty" end of the toothpick usually does not come into contact with the medium This method may be used when picking plaques that will be used immediately (e g , to generate templates for sequencing), but it is not recommended when picking plaques that will be stored for long periods of time or that will be used to make master stocks of valuable recombinants

3. Let the suspension stand for 1 to 2 hours at room temperature to allow the bacteriophage particles to diffuse out of the agar An average plaque contains between 10^6 and 10^8 pfu. The suspension of bacteriophage particles can be stored indefinitely in LB medium at 4°C or −20°C without loss of viability.

Note

Bacteriophage M13 can diffuse considerable distances through top agar. To minimize the possibility of cross contamination, it is therefore important to pick plaques that are well-separated (~1 cm) from their nearest neighbors Do not pick plaques that have been grown for more than 12 hours at 37°C or stored for extended periods at 4°C

PREPARING STOCKS OF BACTERIOPHAGE M13 FROM SINGLE PLAQUES

Stocks of bacteriophage M13 are usually grown in liquid culture. The infected bacteria do not lyse, but instead grow slowly to form a dilute suspension. The inoculum for the bacteriophage stock is almost always a freshly picked plaque or a suspension of bacteriophage particles obtained from a single plaque.

Liquid Cultures

1. Add 50 μl of a culture of plating bacteria (see page 4.21) to 2 ml of LB medium in a sterile 15-ml culture tube.

2. Either add one tenth of a suspension of bacteriophage particles derived from a single plaque (see page 4.24) or touch the surface of a plaque with a sterile, 8-cm-long wooden stick (sometimes called applicator or orange stick). Wash the end of the stick in the medium containing the plating bacteria.

3. Incubate the infected culture for either 4–5 hours (entire plaque) or 5–6 hours (one tenth of a plaque suspension) at 37°C with constant agitation. To minimize the possibility of selecting deletion mutants, cultures infected with recombinant M13 bacteriophages should not be grown for longer periods of time.

4. Transfer 1 ml of the culture to a sterile microfuge tube and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge. Transfer the supernatant to a fresh, sterile microfuge tube, being careful not to disturb the pellet of bacteria. The stock can be stored indefinitely at 4°C or −20°C without loss of infectivity. The titer of the stock should be $\sim 10^{12}$ pfu/ml. The remaining 1 ml of the infected bacterial culture may be used to prepare single-stranded DNA or double-stranded RF DNA (see pages 4.26–4.30).

PREPARATION OF BACTERIOPHAGE M13 DNA

Bacteria infected with bacteriophage M13 contain the double-stranded replicative form of bacteriophage M13 DNA and produce virus particles containing single-stranded progeny DNA. Amounts of both forms of the viral DNA sufficient for most purposes may be isolated from small-scale cultures of infected bacteria by the same methods used to prepare plasmid DNA.

Small-scale Preparations of the Replicative Form of Bacteriophage M13 DNA

1. Prepare an infected culture (2 ml) as described on page 4.25. To minimize the possibility of selecting deletion mutants, cultures infected with recombinant M13 bacteriophages should be grown for the shortest possible time required to achieve an adequate yield of DNA. For most of the vector/host combinations in current use, cultures grown for 4.0–4.5 hours at 37°C will yield ~500 ng of RF DNA per milliliter.

   If desired, use up to 1 ml of the culture to prepare a master stock of bacteriophage particles. The remainder of the infected culture is used to prepare RF DNA.

2. Transfer 1.0–1.5 ml of the infected culture to a microfuge tube, and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge. The infected bacteria form a visible pellet, and the bacteriophage particles remain in the supernatant.

3. Transfer the supernatant to a fresh, sterile microfuge tube, being careful not to disturb the bacterial pellet, and store at 4°C. If desired, single-stranded bacteriophage M13 DNA may be prepared from this supernatant at a later stage (see page 4.29).

4. Using a micropipette, remove any residual supernatant from the tube containing the bacterial pellet and discard. Resuspend the pellet by vigorous vortexing in 100 μl of ice-cold Solution I.

   Solution I

   50 mM glucose
   10 mM EDTA (pH 8.0)
   25 mM Tris · Cl (pH 8.0)

   Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 10 lb/sq. in. on liquid cycle, and stored at 4°C.

It is essential to ensure that the bacterial pellet is completely dispersed in Solution I. This can be achieved rapidly by vortexing two microfuge tubes simultaneously with their bases touching (see Figure 1.2, page 1.26).

The original protocol called for the use of lysozyme at this point to digest the bacterial cells. This step is unnecessary.

Some strains of bacteria shed into the medium cell-wall components that can inhibit the action of restriction enzymes This problem can be avoided by resuspending the bacterial cell pellet in 0.5 ml of STE (0.1 M NaCl, 10 mM Tris Cl [pH 8.0], 1 mM EDTA [pH 8.0]) and recentrifuging After removal of the STE, resuspend the pellet in Solution I as described above

5. Add 200 μl of freshly prepared Solution II

Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)
1% SDS

Close the tube tightly and mix the contents by inverting the tube rapidly five times Make sure that the entire surface of the tube comes in contact with Solution II Do not vortex Store the tube on ice.

6. Add 150 μl of ice-cold Solution III

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Close the tube and vortex it gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate Store the tube on ice for 3–5 minutes

7. Centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge Transfer the supernatant to a fresh tube

8. Add an equal volume of phenol chloroform Mix by vortexing. After centrifuging at 12,000*g* for 2 minutes in a microfuge, transfer the supernatant to a fresh tube

9. Precipitate the double-stranded DNA with 2 volumes of ethanol at room temperature Mix by vortexing Allow the mixture to stand for 2 minutes at room temperature

10. Centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge

11. Remove the supernatant by gentle aspiration Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube.

The supernatant can be conveniently removed with a disposable pipette tip attached to a vacuum line (see Figure 1 3, page 1 27) Use a gentle vacuum and touch the tip to the surface of the liquid Keep the tip as far away from the pellet of nucleic acid as possible as the fluid is withdrawn from the tube The pipette tip can then be used to vacuum the walls of the tube to remove any adherent droplets of fluid

12. Rinse the pellet of double-stranded DNA with 1 ml of 70% ethanol at 4°C Remove the supernatant as described in step 11, and allow the pellet of nucleic acid to dry in the air for 10 minutes.

13. To remove RNA, resuspend the pellet in 25 μl of TE (pH 7.6) containing DNAase-free pancreatic RNAase (20 μg/ml) (see Appendix B) Vortex briefly The double-stranded RF DNA is now ready for analysis by digestion with restriction enzymes The yield of RF DNA expected from 1 ml of an infected bacterial culture is usually ~500 ng, which is enough for five digests

Note

This method, devised by Birnboim and Doly (1979) and subsequently modified by Ish-Horowicz and Burke (1981), was used originally to isolate plasmid DNA from bacterial cultures. It is our experience that preparations of bacteriophage M13 RF DNA are never as clean as small-scale preparations of plasmid DNA Often, they are contaminated with single-stranded viral DNA that can confuse the pattern of DNA fragments obtained by digestion with restriction enzymes. This problem can be alleviated by analyzing in parallel an aliquot of RF DNA that has not been digested with restriction enzymes. Linear and circular single-stranded viral DNAs (which usually appear as fuzzy bands migrating faster than the double-stranded RF DNA) are not cleaved by most of the restriction enzymes commonly used for cloning in bacteriophage M13, and therefore their migration through agarose gels does not change after incubation with restriction enzymes If the pattern of bands is confusing, fragments of double-stranded DNA with recessed 3′ termini can usually be identified unambiguously by end-labeling (see Chapter 1, pages 1 56–1 57)

Small-scale Preparations of Single-stranded Bacteriophage M13 DNA

1. Prepare an infected culture (2 ml) as described on page 4.25. To minimize the possibility of selecting deletion mutants, cultures infected with recombinant M13 bacteriophages should be grown for the shortest possible time (≤4.5 hours) required to achieve an adequate yield of DNA.

   If desired, use up to 1 ml of the culture to prepare a master stock of bacteriophage particles. The remainder of the infected culture is used to prepare single-stranded DNA.

2. Transfer 1.0–1.5 ml of the infected culture to a microfuge tube, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

3. Transfer about 1.2–1.3 ml of the supernatant to a fresh microfuge tube, and add 200 μl of a solution of 20% polyethylene glycol (PEG 8000) in 2.5 M NaCl. Mix by inverting the tube several times, followed by gentle vortexing. Let the tube stand for 15 minutes at room temperature.

   The solution of polyethylene glycol in 2.5 M NaCl should be sterilized by filtration. It may then be stored indefinitely at room temperature.

4. Recover the precipitated bacteriophage particles by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

5. Carefully remove all of the supernatant using a disposable pipette tip as described on pages 4.27–4.28. Recentrifuge the tube for 30 seconds and remove any residual supernatant. A tiny pellet of precipitated bacteriophage particles should be visible at the bottom of the tube.

   If no pellet is visible, it is unlikely that sufficient single-stranded DNA will be obtained to justify proceeding further. In this case, pick more plaques and grow the infected bacteria for 6 hours.

   Contamination of the bacteriophage pellet with components in the supernatant causes problems in dideoxy sequencing reactions, and it is therefore important to take special care to remove all traces of supernatant from the microfuge tube.

6. Resuspend the pellet in 100 μl of TE (pH 8.0) by vigorous vortexing.

   A multitube vortexing machine (Baxter SMI, 2600) saves much time and effort when many samples are to be processed simultaneously.

7. Add 50 μl of phenol (not phenol:chloroform) equilibrated with Tris · Cl (pH 8.0) (see Appendix B). Mix well by vortexing for 30 seconds. Allow the sample to stand for 1 minute at room temperature, and then vortex for another 30 seconds.

8. Centrifuge the sample at 12,000*g* for 1 minute at room temperature in a microfuge. Using an automatic pipettor, transfer the upper, aqueous phase to a fresh microfuge tube containing 300 μl of a 25:1 mixture of absolute ethanol:3 M sodium acetate (pH 5.2). Vortex briefly. Store for 15 minutes at room temperature.

Do not try to transfer all of the aqueous phase Much cleaner preparations of single-stranded DNA are obtained when ~5 μl of the aqueous phase is left at the interface

Some investigators extract the aqueous phase with chloroform before precipitating the DNA with ethanol. Single-stranded DNAs made in this way are the best templates for DNA sequencing reactions However, the results obtained from templates prepared with phenol alone are adequate for most purposes, and there is normally no need to use chloroform

9. Recover the precipitated single-stranded bacteriophage DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge Remove the supernatant by gentle aspiration, being careful not to disturb the pellet (which is often barely visible) Recentrifuge the tube for 15 seconds and remove any residual supernatant.

10. Add 200 μl of 70% ethanol at room temperature, vortex briefly, and centrifuge. Immediately remove the supernatant by gentle aspiration At this stage, the pellet is not attached quite so firmly to the wall of the tube, and it is therefore important to work quickly and carefully to avoid losing the DNA.

11. Stand the open tube on the bench for 10 minutes to allow any residual ethanol to evaporate Dissolve the pellet in 50 μl of TE (pH 8 0) Store at −20°C. The yield of single-stranded DNA is usually 5–10 μg per milliliter of the original infected culture

Large-scale Preparations of the Replicative Form of Bacteriophage M13 DNA

1. Prepare a small stock of the desired bacteriophage for use as an inoculum (see page 4.25).

2. Transfer 2.5 ml of plating bacteria (see page 4.21) to a sterile tube (13 mm × 100 mm). Add 0.5 ml of the bacteriophage stock. Mix by tapping the side of the tube. Allow the tube to stand for 5 minutes at room temperature.

3. Dilute the infected cells into 250 ml of fresh LB medium, prewarmed to 37°C, in a 2-liter flask. Incubate for 5 hours at 37°C with constant, vigorous shaking.

4. Harvest the infected cells by centrifugation at 4000*g* for 15 minutes at 4°C. Carefully remove the supernatant (which may be used for large-scale preparations of single-stranded bacteriophage M13 DNA, if desired).

5. Resuspend the bacterial pellet in 100 ml of ice-cold STE. Recover the washed cells by centrifugation at 4000*g* for 15 minutes at 4°C.

   STE

   0.1 M NaCl
   10 mM Tris·Cl (pH 8.0)
   1 mM EDTA (pH 8.0)

6. Isolate the bacteriophage M13 closed circular RF DNA by the alkaline lysis method (see Chapter 1, page 1.38) and purify by precipitation with polyethylene glycol or by equilibrium centrifugation in CsCl–ethidium bromide gradients (see Chapter 1, page 1.40 or 1.42). The yield of RF DNA expected from 250 ml of infected cells is ~200 μg.

Large-scale Preparations of Single-stranded Bacteriophage M13 DNA

1. Transfer the supernatant set aside in step 4 (page 4.31) to a 500-ml beaker containing a magnetic stirring bar.

2. Add 10 g of polyethylene glycol (PEG 8000) and 7.5 g of NaCl to the supernatant, and stir for 30 minutes to 1 hour at room temperature. Stirring for longer periods of time can result in the precipitation of undesired bacterial debris.

3. Collect the precipitate by centrifugation at 10,000*g* for 20 minutes at 4°C. Let the centrifuge bottle stand in an inverted position for 2–3 minutes to allow the supernatant to drain away from the precipitate. Use Kimwipes to remove the last traces of supernatant from the walls and neck of the bottle.

4. Add 10 ml of a solution of 10 mM Tris·Cl (pH 8.0) to the bottle. Use a pasteur pipette to collect the precipitate (which is smeared over the wall of the bottle) and to transfer it to a 30-ml Corex centrifuge tube.

5. Add an equal volume of phenol equilibrated with Tris·Cl (pH 8.0) (see Appendix B). Seal the tube, and mix the contents by vortexing vigorously for 2 minutes.

6. Centrifuge at 3000*g* for 5 minutes at room temperature.

7. Transfer the upper, aqueous phase to a fresh tube and repeat the extraction, this time using phenol.chloroform equilibrated with Tris·Cl (pH 8.0).

   If there is a visible interface, extract the supernatant once more with chloroform.

8. Transfer the upper, aqueous phase to a clean 50-ml tube. Add 1 ml of 3 M sodium acetate (pH 5.2) and 22 ml of absolute ethanol. Mix well and store at 0°C for 1 hour.

9. Recover the precipitate of single-stranded DNA by centrifugation at 12,000*g* for 20 minutes at 4°C.

10. Carefully discard all of the supernatant. Add 30 ml of 70% ethanol at 4°C to the tube. Vortex briefly.

11. Centrifuge at 12,000*g* for 10 minutes at 4°C. Carefully remove as much of the supernatant as possible, and store the tube in an inverted position to allow the last traces of supernatant to drain away from the precipitate. Watch carefully to make sure that the precipitate does not slide out of the tube. Wipe the neck of the tube with Kimwipes.

12. Allow the pellet to dry at room temperature, and then dissolve it in 1 ml of TE (pH 8.0). Store the DNA at −20°C. The yield of single-stranded DNA expected from 250 ml of infected culture is 250 μg to 1 mg.

Cloning into Bacteriophage M13 Vectors and Transfection of Competent Bacteria

Fragments of foreign DNA are inserted into bacteriophage M13 vectors in exactly the same way that they would be inserted into a conventional plasmid Four different methods are commonly used:

1 The polycloning site of the vector is cleaved with a single restriction enzyme, and the resulting linear DNA is ligated with an excess of foreign DNA that has compatible termini. No effort is made to reduce the background of nonrecombinant molecules formed by recircularization of the vector Bacteriophages carrying foreign DNA sequences are identified by their ability to form colorless plaques when plated in media containing IPTG and X-gal Recircularized, nonrecombinant bacteriophages form blue plaques The foreign DNA can be carried in such recombinant bacteriophages in two possible orientations.

2 An additional step is included in method 1 above After linearization, the vector is treated with phosphatase to reduce its ability to recircularize during ligation (see Chapter 1, page 1 60) The foreign DNA can be carried in such recombinant bacteriophages in two possible orientations

3 The polycloning site of the vector is cleaved with two different restriction enzymes, generating linear molecules with incompatible termini (see Chapter 1, pages 1 53–1 56) The linear vector is ligated to an excess of foreign DNA that has termini compatible with those of the vector In this type of forced directional cloning, no effort is made to reduce the background of nonrecombinant molecules formed by reinsertion of the small fragment of the polycloning site into the vector Bacteriophages carrying foreign DNA sequences are identified by their ability to form colorless plaques when plated in media containing IPTG and X-gal The foreign DNA can be inserted into the vector in only one orientation

4 An additional step is included in method 3 above After cleavage of the vector DNA, the small fragment of the polycloning site is removed by electrophoresis through an agarose or polyacrylamide gel (see Chapter 6) or by spun-column chromatography through Sepharose CL-4B (see Appendix E) This reduces the number of nonrecombinant clones created by reinsertion of the small fragment of the polycloning site into the vector Foreign DNA sequences can be inserted into the vector in only one possible orientation

Which of these four methods should be used is partly a matter of personal preference, but there are several factors to consider when deciding among them

- Methods 1 and 3 should be used only if sufficient foreign DNA is available to allow the ligation reaction to be set up with a three- to tenfold molar excess of the foreign DNA fragment. If the amount of foreign DNA is limited, use method 2 or 4

- If the replicative form of the vector DNA has been cleaved with two different restriction enzymes, the fragment of foreign DNA can be inserted in only one orientation with respect to the surrounding bacteriophage M13 sequences This usually causes no problems when the final recombinant is to be used to generate single-stranded templates for site-directed mutagenesis However, directional cloning may impose limitations when the single-stranded templates are to be used to determine the sequence of the foreign DNA or to generate strand-specific probes If "universal primer" is used in the reaction, the sequences obtained will be restricted to the 300–500 nucleotides of foreign DNA immediately downstream from the primer-binding site; radioactive probes will correspond to only one of the strands of the foreign DNA. One solution to this problem is to insert the foreign DNA separately into each member of a pair of bacteriophage M13 vectors (e g., M13mp18 and M13mp19) that carry the polycloning site in opposite orientations Single-stranded templates prepared from these recombinants will yield the terminal sequences of each of the two strands of the foreign DNA, radioactive probes can be generated that will hybridize specifically to one strand or the other of the foreign DNA
- When the DNAs are to be cleaved with two restriction enzymes (methods 3 and 4), the digestions can be carried out simultaneously if both enzymes work in the same buffer Alternatively, the enzyme that works in the buffer of lower ionic strength should be used first. After incubation for an appropriate time, analyze a small sample of the DNA in the reaction by agarose gel electrophoresis If the digestion is complete, adjust the ionic strength of the buffer, add the second enzyme, and continue incubation. Then set up a pilot reaction (10 μl) containing the second restriction enzyme, 50–100 ng of a test DNA that contains more than one site for the second enzyme, and 9 μl of the large-scale reaction After incubation, analyze the test DNA in the pilot reaction by agarose gel electrophoresis. If the reaction is complete, purify the DNA in the large-scale reaction by extraction with phenol chloroform and precipitation with ethanol
- Because bacteriophage M13 is not a suitable vector either for the routine propagation of foreign DNA segments or for sequencing or mutagenesis of large segments of foreign DNA, special efforts should be made to construct recombinants containing the smallest possible fragment of foreign DNA that carries the sequences of interest

Whichever method is used to prepare the vector DNA, a sample of the final preparation should be analyzed by electrophoresis through an agarose gel to check the integrity of the DNA If the vector is to be used routinely to clone different segments of foreign DNA, it is advisable to set up a series of test ligations and transformations to measure the background and to determine the efficiency of the vector.

Cloning DNA Fragments into Bacteriophage M13 Vectors

1. Mix together appropriate amounts of vector DNA, prepared by one of the methods described above, and the fragment(s) of foreign DNA to be cloned The combined volume of the two DNAs should not exceed 8 μl. If necessary, add TE (pH 8.0) to bring the volume to 7 5 μl. Add 1 μl of 10 × ligation buffer and 1 μl of 10 mM ATP Place 1 μl of the mixture into a microfuge tube containing 9 μl of TE (pH 8 0), and store at 4°C for later analysis by gel electrophoresis

   For each 10 μl of ligation reaction, a combination of 20–40 ng of vector DNA and a three- to tenfold molar excess of foreign DNA will produce an adequate yield of recombinant genomes for most cloning purposes

   10 × Ligation buffer
   0.5 M Tris · Cl (pH 7 6)
   100 mM $MgCl_2$
   100 mM dithiothreitol
   500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

2. Set up two control reactions containing

   a the same amount of vector DNA, but no foreign DNA

   b the same amount of vector DNA and an appropriate amount of a test DNA that has been successfully cloned into bacteriophage M13 on a previous occasion (e g , bacteriophage λ DNA cleaved with restriction enzymes that recognize tetranucleotide sequences and generate appropriate termini)

3. Add 0 5 Weiss unit of bacteriophage T4 DNA ligase to each of the reactions from steps 1 and 2, and mix by vortexing briefly (For definition of Weiss unit, see Chapter 5, page 5 62) Incubate for 4–6 hours at 16°C

4. After ligation, transfer an aliquot (1 μl) from each ligation to a microfuge tube containing 9 μl of TE (pH 8 0) Analyze these aliquots, and the unligated aliquot of the mixture set aside in step 1, by electrophoresis through a 0 7% agarose gel to check that the ligation reaction has been successful

   The kinetics of ligation can often be improved by
   - coprecipitating the fragments of DNA with ethanol before setting up the reaction,
   - including 5% polyethylene glycol (PEG 8000) in the ligation reaction (Pheiffer and Zimmerman 1983, Zimmerman and Harrison 1985), or
   - including hexamminecobalt chloride in the reaction (Rusche and Howard-Flanders 1985, Tessier et al 1986)

   The effects of these compounds on the ligation of DNA are discussed in more detail in Chapter 1, pages 1 70–1 71

5. Introduce 5 μl of each of the remaining samples into competent bacteria of the appropriate strain of *E coli* (see page 4 37)

Preparation of Competent Bacteria

1. Streak a master culture of bacterial strain TG1 or TG2 onto a minimal agar plate. Incubate for 24–36 hours at 37°C.

   The genes coding for enzymes involved in proline biosynthesis have been deleted from the chromosomes of TG1 and TG2. Thus, only those bacteria that carry the F′ plasmid (F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) will be able to form colonies on media lacking in proline Bacteria grow much more slowly on minimal media than on rich media, and they do not survive prolonged periods of storage at 4°C Master stocks of TG1 and TG2 carrying F′ episomes should therefore be stored at −70°C in LB medium containing 15% glycerol (see Appendix A)

   Other strains (e g , JM101 and JM109) can be used to prepare bacteria that are competent for transfection by bacteriophage M13 DNA However, TG1 and TG2 have the advantage of combining a set of desirable genetic markers with the ability to be transfected efficiently with double-stranded viral DNA

   Strain MV1184 (see Table 4 1) should be used for transformation with the phagemids pUC118 and pUC119 (see page 4 44)

2. Prepare a batch of frozen competent bacteria as described in Chapter 1, pages 1.76–1 81

Transfection of Competent Bacteria with Bacteriophage M13 DNA

1. Grow an overnight culture of plating bacteria (TG1 or TG2) in SOB medium at 37°C with constant shaking

2. Remove an aliquot of frozen competent TG1 or TG2 cells from the −70°C freezer Allow it to thaw at room temperature, and then place on ice for 10 minutes

3. Transfer 50 μl of the competent bacteria to each of a series of sterile 5-ml culture tubes (Falcon 2054) that have been chilled to 0°C. Immediately add 5 μl of each of the ligation reactions (page 4 35) to separate tubes. Two controls should be included, one containing 5 pg of bacteriophage M13 double-stranded circular DNA and the other no DNA at all Mix the DNAs with the bacteria by tapping the sides of the tubes gently Store on ice for 30 minutes

   Tubes other than Falcon 2054 may be used, but in this case it will be necessary to determine empirically the optimum time and temperature of heat shock

4. Prepare a set of sterile culture tubes containing 3 ml of melted SOB top agar Store at 47°C until needed in step 6

5. Transfer the tubes containing the competent bacteria and DNA to a water bath equilibrated at 42°C Incubate for exactly 90 seconds Immediately return the tubes to an ice bath After 2 minutes, add 175 μl of SOB medium lacking magnesium Mix by gentle vortexing.

6. Add 40 μl of a solution of X-gal (20 mg/ml in dimethylformamide) and 4 μl of a solution of IPTG (200 mg/ml) to the tubes containing the melted SOB top agar prepared in step 4, and mix by vortexing Transfer aliquots of the transfected bacteria (1 μl, 10 μl, and 100 μl) to the tubes Immediately add 200 μl of the overnight culture of TG1 or TG2 cells (step 1) Mix by vortexing Pour the mixture onto a labeled LB agar plate Swirl the plate gently to ensure an even distribution of bacteria and top agar

   Solutions of IPTG and X-gal are prepared and stored as described on page 4 22

7. Close the plates and allow the top agar to harden for 5 minutes at room temperature. Invert the plates and incubate at 37°C Plaques will begin to appear after 4 hours and will be fully developed after 8–12 hours

   Plaques formed by wild-type bacteriophage M13 will be deep blue, those formed by recombinants will be colorless

Note

The numbers of blue and colorless plaques obtained depend on many factors, including the following·

- Whether the DNA of the bacteriophage M13 vector was cleaved by one or two different restriction enzymes

- The stoichiometry of the DNAs in the ligation reactions
- The efficiency with which the competent bacteria were transfected with the viral DNAs

Typical results might be.

	Number of plaques/ μg vector DNA	
RF DNA	**blue**	**colorless**
Closed circular	3×10^7	3×10^3[a]
Linearized by cleavage with one restriction enzyme	1×10^5	$\sim 5 \times 10^4$
Linearized by cleavage with one restriction enzyme and self-ligated	2×10^7	$\sim 1 \times 10^4$
Linearized by cleavage with one restriction enzyme, phosphatase-treated, and self-ligated	3×10^4	$\sim 5 \times 10^4$
Cleaved by two different restriction enzymes	$< 5 \times 10^3$	$\sim 1 \times 10^4$
Linearized by cleavage with one restriction enzyme and ligated in the presence of a tenfold molar excess of foreign DNA cleaved with a compatible restriction enzyme	$\sim 1 \times 10^5$	$\sim 4 \times 10^6$
Cleaved by two different restriction enzymes and ligated in the presence of a tenfold molar excess of a fragment of foreign DNA carrying compatible termini	$< 5 \times 10^3$	$\sim 2 \times 10^6$

[a] The small proportion of colorless plaques is formed by spontaneous mutants that have lost the ability to accomplish α-complementation

Identification and Analysis of Recombinants

Insertion of foreign DNA sequences into bacteriophage M13 vectors almost invariably causes inactivation of the amino-terminal fragment of β-galactosidase. Although a small number of recombinants have been identified that continue to display α-complementation (see, e g , Close et al 1983), the great majority of recombinant M13 bacteriophages form pale blue or colorless plaques when plated in media containing IPTG and X-gal (Gronenborn and Messing 1978) Several methods are available to analyze the foreign DNA sequences in these colorless plaques

Direct Gel Electrophoresis

Recombinants carrying sequences of foreign DNA longer than 200–300 nucleotides can be detected by gel electrophoresis of DNA released from infected bacteria

1. Prepare stocks of putative recombinant bacteriophages from single plaques as described on page 4 25 As controls, prepare stocks of several nonrecombinants (picked from well-isolated blue plaques)

2. Transfer 1 5 ml of the infected cell cultures to microfuge tubes, and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge.

3. Using an automatic pipetting device equipped with a sterile tip, transfer 20 μl of each of the supernatants into fresh microfuge tubes Store the remainders of the cultures at 4°C until needed. Add 1 μl of 2% SDS, mix by vortexing briefly, and incubate at 65°C for 5 minutes Add 5 μl of gel-loading buffer (see Appendix B), mix, and load each sample into a well of a 0 7% agarose gel cast in 0 5 × TBE (see Appendix B) and containing ethidium bromide (0.5 μg/ml)

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described in Appendix E

4. After electrophoresis, examine the gel by ultraviolet light and photograph the gel as described in Chapter 6, page 6 19 Compare the electrophoretic mobilities of the single-stranded DNAs liberated from the putative recombinants with those of the DNAs liberated from the control nonrecombinant bacteriophages Recombinants carrying sequences of foreign DNA longer than 200–300 nucleotides migrate more slowly through the gel. Single-stranded DNA can be prepared from the remainders of the original cultures that were stored at 4°C

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

Note

If necessary, the presence of foreign DNA sequences can be confirmed by transferring the single-stranded DNAs from the gel to a nitrocellulose filter or nylon membrane and hybridizing to an appropriate radiolabeled probe (see Chapter 9, pages 9 34–9 55). Soak the gel in 20 × SSC for 45 minutes, and then transfer the DNA directly to the filter There is no need to denature the DNA by soaking the gel in alkali

In Situ Hybridization of Bacteriophage M13 Plaques

Bacteriophage M13 plaques can be screened by hybridization to ^{32}P-labeled probes using essentially the same methods devised for screening bacteriophage λ (Benton and Davis 1977) Transfer the bacteriophage DNA to a nitrocellulose filter or nylon membrane as described in Chapter 2, pages 2 109–2 111

Note

The bacteriophage M13 DNA transferred to the filter does not need to be denatured with alkali Instead, after removing the filter from the surface of the agar or agarose, allow it to dry (DNA side up) at room temperature Bake the filter under vacuum at 80°C for 2 hours and hybridize to ^{32}P-labeled probes as described in Chapter 2, pages 2 114–2.117 Double-stranded probes will hybridize to all recombinants irrespective of the orientation of the segment of foreign DNA within the vector, single-stranded probes will hybridize only to recombinants that carry complementary sequences attached to the (+) strand of viral DNA

Analysis of Small-scale Preparations of the Replicative Form of Bacteriophage M13 DNA

1. Prepare double-stranded RF DNA from cultures infected with putative recombinants as described on pages 4 26–4 28 As controls, prepare RF DNA from several nonrecombinants (picked from well-isolated blue plaques)

2. Digest aliquots of the nonrecombinant and putative recombinant RF DNAs with appropriate restriction enzymes, and analyze the resulting fragments by electrophoresis through an agarose gel (0 7–2 5%, depending on the expected sizes of the fragments) cast in 0 5 × TBE (see Appendix B) and containing ethidium bromide (0 5 μg/ml). As controls, analyze aliquots of recombinant and nonrecombinant DNAs that have not been digested with restriction enzymes

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described in Appendix E

Note

If necessary, the presence of foreign DNA sequences can be confirmed by transferring the DNAs from the gel to a nitrocellulose filter or nylon membrane and hybridizing to an appropriate radiolabeled probe (see Chapter 9, pages 9.34–9 55)

Testing the Orientation of DNA Cloned in Bacteriophage M13 Vectors

If the polycloning site in the vector has been cleaved with a single restriction enzyme and ligated to foreign DNA that has compatible termini, there is an opportunity for the foreign DNA to become inserted into the vector in either orientation. The (+) strand of the resulting recombinants may therefore contain complementary sequences of foreign DNA. There are two rapid ways to identify recombinants that carry foreign sequences in opposite orientations. (1) Screen the recombinant DNAs with single-stranded probes of known polarity (see Chapter 10 for methods to prepare probes) or (2) using the method below (Gardner et al. 1981, Howarth et al. 1981), test for the ability of the (+) strands of different recombinants to hybridize to one another

1. Prepare stocks of putative recombinants from single plaques as described on page 4 25. As controls, prepare stocks of several nonrecombinants (picked from well-isolated blue plaques)

2. Transfer 1 5 ml of the infected cell cultures to microfuge tubes, and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge.

3. Mix 20 μl of the supernatants of two different recombinants in fresh 0 5-ml microfuge tubes Set up several tubes containing different recombinants in pairwise combinations As controls, transfer 40 μl of the supernatants of one or two individual recombinants into separate tubes Store the remainders of the cultures at 4°C until needed.

4. Add 2 μl of 2% SDS to the tubes containing the samples of the supernatants Mix by vortexing briefly, and incubate for 5 minutes at 65°C Add 4 μl of 0 5 M NaCl, mix well, and then continue the incubation for another hour at 65°C

   If evaporation is a problem during incubation at 65°C, cover the fluid in the microfuge tubes with a thin layer of paraffin oil

5. Add 5 μl of gel-loading buffer (see Appendix B) to the aqueous phase, mix, and load each sample into a well of a 0 7% agarose gel cast in 0 5 × TBE (see Appendix B) and containing ethidium bromide (0 5 μg/ml)

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described in Appendix E

6. After electrophoresis, examine the gel by ultraviolet light and photograph the gel as described in Chapter 6, page 6.19 If the recombinants contain complementary sequences, they will hybridize to form figure-eight structures that migrate more slowly through the gel than does single-stranded viral DNA

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

Note

This procedure can also be used to determine the sizes of fragments of foreign DNA cloned in bacteriophage M13 (Aegerter and Trachsel 1987) After the annealing of bacteriophage DNAs containing complementary sequences (step 4), add 5 μl of 10 × nuclease-S1 buffer and 1 unit of nuclease S1. Incubate for 30 minutes at 37°C, and then analyze the samples by electrophoresis through an agarose gel (0 7–2 5%, depending on the expected sizes of the fragments)

10 × Nuclease-S1 buffer

2 M NaCl
0.5 M sodium acetate (pH 4.5)
10 mM $ZnSO_4$
5% glycerol

The size of the nuclease-S1-resistant double-stranded DNA is a measure of the length of the complementary sequences carried in opposite orientations in the bacteriophage M13 recombinants

Cloning in Phagemids

In the phagemid system devised by Vieira and Messing (1987), foreign DNA is cloned by conventional techniques into plasmids pUC118 and/or pUC119 and propagated as double-stranded DNA pUC118 and pUC119 are derivatives of pUC18 and pUC19 that contain a 476-bp fragment derived from the intergenic region of wild-type bacteriophage M13 (from the *Hgi*AI site at nucleotide 5465 to the *Dra*I site at nucleotide 5941) inserted at the unique *Nde*I site of pUC18 and pUC19 (nucleotide 2499). Infection of cells carrying the phagemid with the helper virus M13KO7 results in the production of bacteriophage particles containing single-stranded copies of the phagemid genome. The intergenic region of the phagemid is oriented so that the progeny virus particles contain the same strand of the *lac* region as bacteriophages of the mp series (Figure 4 6)

M13KO7 is a derivative of bacteriophage M13 that carries a mutated[2] version of gene II (derived from M13mp1), a plasmid origin of replication (derived from p15A [Selzer et al 1983]), and the kanamycin resistance gene from Tn*903* (Grindley and Joyce 1980) at the *Ava*I site (nucleotide 5825 of bacteriophage M13) (Figure 4.7) When M13KO7 infects cells carrying pUC118 or pUC119, the incoming single-stranded DNA is converted by cellular enzymes to a double-stranded form that then uses the plasmid p15A origin to replicate Because the accumulation of double-stranded M13KO7 DNA does not require viral gene products, there is little opportunity for the resident phagemids to interfere with the early stages of replication of the incoming bacteriophage genome.

The pool of double-stranded M13KO7 genomes expresses all of the proteins required to generate progeny single-stranded DNA However, the mutated gene II product encoded by M13KO7 interacts less efficiently with the bacteriophage origin of replication carried on its own genome than with the origin cloned into pUC118 and pUC119 (Dotto and Zinder 1984b). This results in preferential production of (+) strands from the phagemid and ensures that the viral particles produced by the cell contain a preponderance of single-stranded DNA derived from the phagemid When M13KO7 is grown in the absence of pUC118 and pUC119, the mutated gene II protein interacts well enough with the disrupted origin of replication to produce sufficient bacteriophage for use as an inoculum for the production of single-stranded phagemid DNA

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[2] There is a G→T transition at nucleotide 6125 in the sequence of bacteriophage M13 This results in a substitution of an isoleucine for a methionine at amino acid 40 of the gene II protein (Dotto and Zinder 1984b)

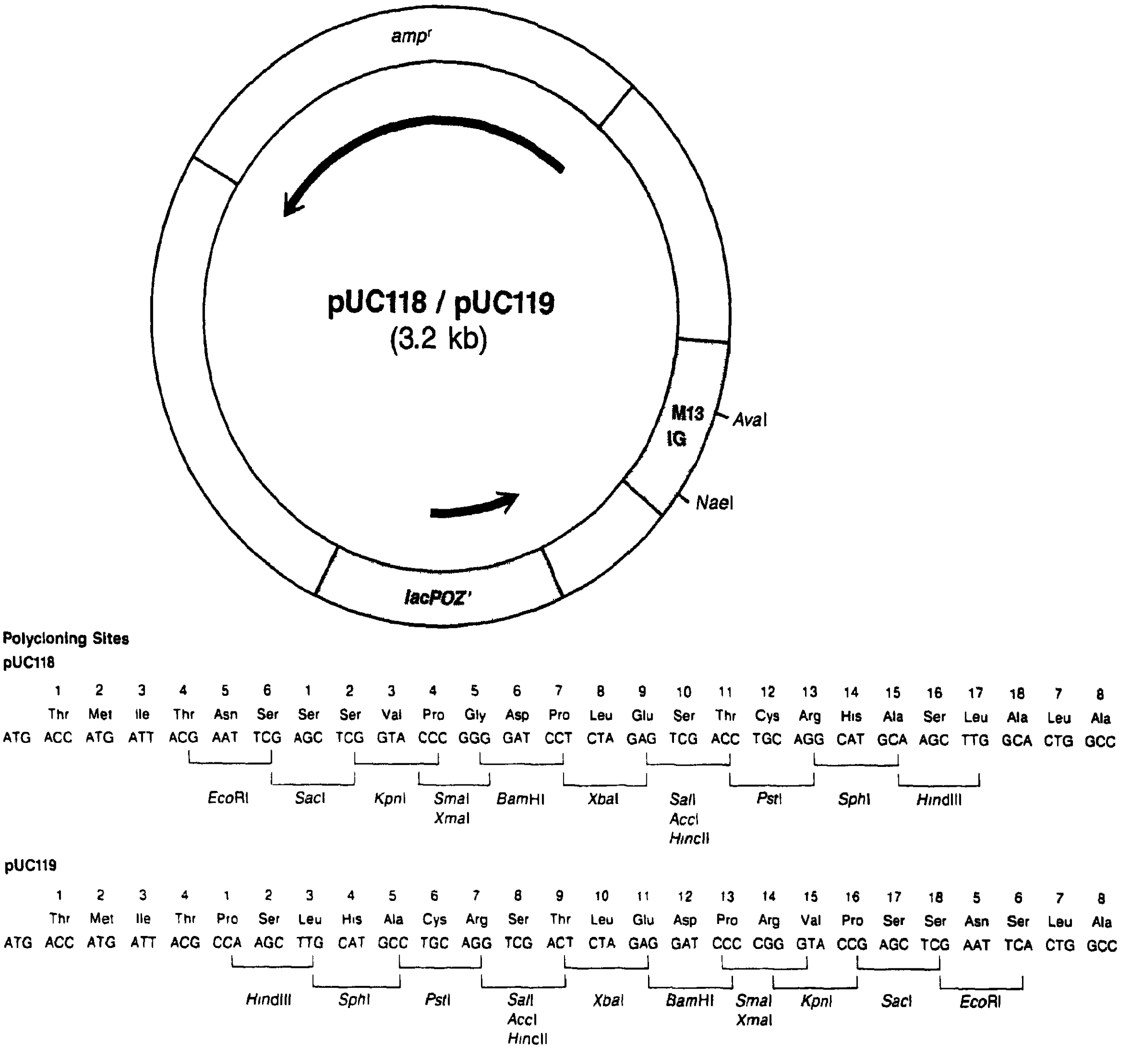
amp^r
pUC118 / pUC119
(3.2 kb)
M13
IG
AvaI
NaeI
lacPOZ'
Polycloning Sites
pUC118
1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 7 8
Thr Met Ile Thr Asn Ser Ser Ser Val Pro Gly Asp Pro Leu Glu Ser Thr Cys Arg His Ala Ser Leu Ala Leu Ala
ATG ACC ATG ATT ACG AAT TCG AGC TCG GTA CCC GGG GAT CCT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG GCA CTG GCC
EcoRI
SacI
KpnI
SmaI
XmaI
BamHI
XbaI
SalI
AccI
HincII
PstI
SphI
HindIII
pUC119
1 2 3 4 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 5 6 7 8
Thr Met Ile Thr Pro Ser Leu His Ala Cys Arg Ser Thr Leu Glu Asp Pro Arg Val Pro Ser Ser Asn Ser Leu Ala
ATG ACC ATG ATT ACG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT TCA CTG GCC
HindIII
SphI
PstI
SalI
AccI
HincII
XbaI
BamHI
SmaI
XmaI
KpnI
SacI
EcoRI

FIGURE 4.6

Phagemid vectors pUC118 and pUC119 The positions of the unique sites in the 476-bp intergenic region are shown Restriction sites in the remainder of the vector are the same as in pUC18 and pUC19

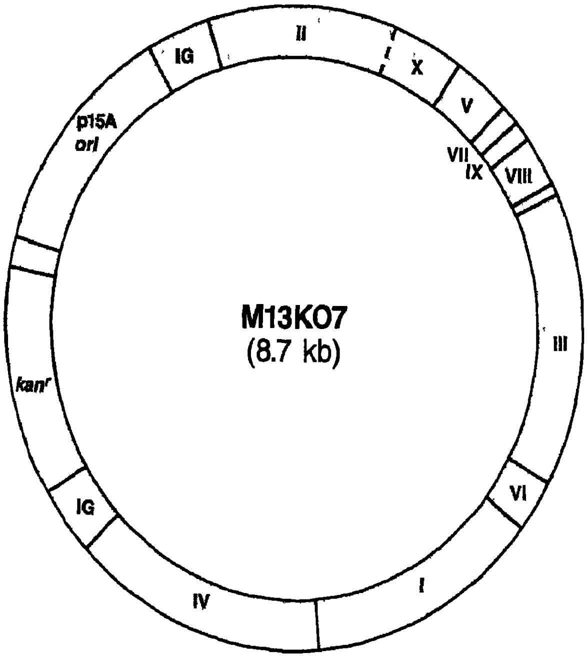

FIGURE 4.7
The genetic map of M13KO7 (Vieira and Messing 1987)

Growth of M13KO7

1. Grow a culture of *E. coli* strain MV1184 (see Tables 4.1 and 4 2) in 2 × YT medium until the OD_{600} reaches 0 8.

2. Prepare a series of tenfold dilutions of M13KO7 in 2 × YT medium, and plate out aliquots of the bacteriophage to obtain well-isolated plaques on a lawn of MV1184 cells as described on page 4 22

   Vieira and Messing (1987) suggest a more economical method in which a stock of M13KO7 is streaked onto the surface of a YT agar plate Top agar (4 ml in 2 × YT medium) containing 0 5 ml of the culture of MV1184 is then poured across the plate from the more dilute side of the streak to the more concentrated side Plaques appear after 8–10 hours of incubation at 37°C

3. Pick well-separated, single plaques and place each plaque in 2–3 ml of 2 × YT medium containing kanamycin (70 μg/ml) in a 15-ml culture tube. Incubate for 12–16 hours at 37°C with moderate agitation (250 cycles/minute).

4. Transfer the cells to a sterile microfuge tube, and centrifuge at 12,000*g* for 2 minutes at 4°C in a microfuge Transfer the supernatant to a fresh microfuge tube and store at 4°C Measure the titer of the bacteriophage stock by plaque formation on *E coli* strain JM109 (or another strain of *E coli* that supports the growth of bacteriophage M13).

Notes

i During propagation of M13KO7, there is selection for bacteriophage genomes that have lost the p15A origin and the Tn*903* transposon. It is therefore important not to pass the bacteriophage serially and to use for superinfection stocks of M13KO7 derived directly from a single plaque

ii The titer of particles containing single-stranded plasmid DNA normally exceeds 10^{11} per milliliter of bacterial culture

Production of Single-stranded Phagemid DNA

1. Suspend a fresh bacterial colony containing pUC118 or pUC119 or their derivatives in a sterile 15-ml culture tube containing 2–3 ml of 2 × YT medium Add M13KO7 to a final concentration of 2×10^7 pfu/ml. Incubate for 1.0–1.5 hours at 37°C with strong agitation (300 cycles/minute).

   The bacterial culture should be only slightly turbid at this stage If growth is too vigorous, dilute the culture with prewarmed 2× YT medium until the turbidity is only just visible

2. Add kanamycin (25 mg/ml in H_2O) to a final concentration of 70 μg/ml. Continue incubation for a further 14–18 hours at 37°C

   The yield of virus particles containing single-stranded copies of the plasmid can be measured by infecting MV1184 (see Table 4 1) cells with dilutions of the virus stock and then plating the infected cells on YT agar containing ampicillin (100 μg/ml) The number of ampicillin-resistant colonies that arise after 24 hours incubation at 37°C is a measure of the number of virus particles in the stock that contain single-stranded plasmid DNA (normally 2×10^{11} to 5×10^{11}/ml)

   To obtain the maximum yield of bacteriophage, the infected cultures should be well-aerated

3. Prepare single-stranded bacteriophage M13 DNA as described on page 4 29

Note

This method works well only with fresh bacterial colonies, i e , colonies that have been grown for 18 hours or less and have not been stored at 4°C Older colonies or colonies that have been stored at 4°C should be treated as follows

1 Inoculate 2 ml of 2× YT medium containing ampicillin (100 μg/ml) with a bacterial colony containing pUC118 or pUC119 or recombinants derived from them

2 Incubate at 37°C with strong agitation (300 cycles/minute) until the culture reaches saturation

3 Dilute 20 μl of the saturated culture into 2 ml of 2× YT medium containing M13KO7 at a concentration of 2×10^8 to 4×10^8 pfu/ml

4 Incubate for 1 hour at 37°C with strong agitation

   The bacterial culture should be only slightly turbid at this stage If growth is too vigorous, dilute the culture with prewarmed 2× YT medium until the turbidity is only just visible

5. Proceed as described in steps 2 and 3 of the procedure above

Screening Colonies by Superinfection

Several colonies can be screened simultaneously for their ability to yield single-stranded DNA after superinfection.

1. Suspend a fresh bacterial colony containing pUC118 or pUC119 or their derivatives in a sterile 15-ml culture tube containing 2–3 ml of 2 × YT medium Add M13KO7 to a final concentration of 2×10^7 pfu/ml Incubate for 4 0–4.5 hours at 37°C with strong agitation (300 cycles/minute)

2. Add kanamycin (25 mg/ml in H_2O) to a final concentration of 70 μg/ml. Continue incubation for a further 14–18 hours at 37°C

3. Centrifuge the culture at 12,000*g* for 2 minutes at room temperature Transfer the supernatant to a fresh tube

4. With an automatic pipetting device equipped with a sterile tip, transfer 40 μl of the supernatant into a fresh microfuge tube Store the remainder of the culture at 4°C until needed Add 2 μl of 2% SDS, mix by vortexing briefly, and incubate for 5 minutes at 65°C Add 5 μl of gel-loading buffer (see Appendix B), mix, and load each sample into a well of a 0.7% agarose gel cast in 0 5 × TBE (see Appendix B) and containing ethidium bromide (0 5 μg/ml)

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated using one of the methods described in Appendix E

5. After electrophoresis, examine the gel by ultraviolet light and photograph.

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

6. Using the method described on page 4.29, isolate single-stranded DNA from the cultures that have produced the highest yield of bacteriophage particles

Note

In principle, it should be possible to produce bacteriophage particles that contain single-stranded copies of plasmid DNA in a strain of male *E coli* carrying any plasmid with an appropriate M13 origin of replication. Unfortunately, however, the yield of single-stranded DNA can be greatly affected by the structure and type of the plasmid carried in the cell (Vieira and Messing 1987) For example, M13KO7 is reported to produce very low yields of single-stranded DNA from cells carrying a plasmid derived from pBR322. Similarly, low yields have been obtained from cells carrying derivatives of pUC in which the intergenic region of bacteriophage M13 has been inserted into the polycloning site. Furthermore, although many sequences of foreign

DNA cloned into pUC118 or pUC119 are copied efficiently into single-stranded DNA, others appear to suppress the yield of bacteriophage particles. Finally, Vieira and Messing (1987) point out that the yield of single-stranded plasmid DNA is affected by the bacterial strain used to propagate the plasmid. MV1184 (derived from JM83; see Table 4.1) and MV1190 (derived from JM101) produce satisfactory yields, whereas MV1304 (derived from JM105) does not JM109 carrying pUC118 or pUC119 or plasmids derived from them undergoes significant lysis when infected by M13KO7. Until the reasons for these effects are understood, we recommend using only *E. coli* strain MV1184 as a host to derive single-stranded copies of plasmids derived from pUC118 or pUC119 We also recommend carrying out a series of pilot experiments to determine the yield of single-stranded DNA from infected cells carrying derivatives of pUC118 or pUC119 into which portions of the sequences of interest have been cloned in different orientations.

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5

Enzymes Used in Molecular Cloning

The primary tools used by the molecular biologist in manipulating DNA are restriction enzymes and other DNA/RNA modifying enzymes. Over the years, the number and uses of these enzymes have increased as new molecules have been discovered, our understanding of the details of the enzymatic reactions has improved, and many commercial manufacturers of products supporting molecular cloning have emerged to guarantee the widespread availability of these reagents With these advances, the task of molecular cloning has been both simplified and expanded in scope In this chapter, we describe many of the properties and uses of these important components in molecular cloning procedures.

Restriction and DNA Methylation Enzymes

Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition sequence These enzymes have been classified into three groups Type I and type III enzymes (see Table 5 1) carry modification (methylation) and ATP-dependent restriction (cleavage) activities in the same protein. Type III enzymes cut the DNA at the recognition site and then dissociate from the substrate However, type I enzymes bind to the recognition sequence but cleave at random sites when the DNA loops back to the bound enzyme Neither type I nor type III restriction enzymes are widely used in molecular cloning

Type II restriction/modification systems are binary systems consisting of a restriction endonuclease that cleaves a specific sequence of nucleotides and a separate methylase that modifies the same recognition sequence A large number of type II restriction enzymes have been isolated (see Roberts 1988 and Table 5 1), many of which are routinely used in molecular cloning

The vast majority of type II restriction enzymes recognize specific sequences that are four, five, or six nucleotides in length and display twofold symmetry (A partial list of restriction enzymes ordered by the length of their recognition sequence is provided in Table 5 2) A few enzymes, however, recognize longer sequences or sequences that are degenerate The location of cleavage sites within the axis of dyad symmetry differs from enzyme to enzyme· Some cleave both strands exactly at the axis of symmetry, generating fragments of DNA that carry blunt ends, others cleave each strand at similar locations on opposite sides of the axis of symmetry, creating fragments of DNA that carry protruding single-stranded termini.

TABLE 5.1 Properties of Restriction Enzymes

Prototype enzyme	Recognition sequence[a]	Commercially available[b]	Commercial isoschizomers[c]	Methylation sensitivity[d]	Compatible ends[e]
(a) *Type II enzymes*					
*Aat*II	GACGT↓C	Y			
*Acc*I	GT↓MKAC	Y			
*Acy*I	GR↓CGYC		*Aha*II, *Bbi*II		*Asu*II, *Cla*I, *Hin*P1I, *Hpa*II, *Mae*II, *Nar*I, *Taq*I
*Afl*II	C↓TTAAG	Y			
*Afl*III	A↓CRYGT	Y			
*Aha*III	TTT↓AAA		*Dra*I		blunt
*Alu*I	AG↓CT	Y			blunt
*Alw*NI	CAGNNN↓CTG	Y		?	
*Apa*I	GGGCC↓C	Y		dcm	
*Apa*LI	G↓TGCAC	Y			
*Asu*I	G↓GNCC	Y	*Cfr*13I, *Nsp*IV, *Sau*961	–	
*Asu*II	TT↓CGAA	Y	*Bst*BI, *Nsp*V		*Acy*I, *Cla*I, *Hin*P1I, *Hpa*II, *Mae*II, *Nar*I, *Taq*I
*Ava*I	C↓YCGRG	Y	*Nsp*III		
*Ava*II	G↓GWCC	Y	*Eco*47I, *Sin*I	dcm	
*Ava*III	ATGCAT	Y	*Eco*T22I, *Nsi*I		*Pst*I
*Avr*II	C↓CTAGG	Y			*Nhe*I, *Spe*I, *Xba*I
*Bal*I	TGG↓CCA	Y		dcm-slow	blunt
*Bam*HI	G↓GATCC	Y	*Bst*I	–	*Bcl*I, *Bgl*II, *Mbo*I, *Xho*II
*Bbv*I	GCAGC (8/12)[f]	Y			
*Bbv*II	GAAGAC (2/6)[f]				
*Bcl*I	T↓GATCA	Y		dam	*Bam*HI, *Bgl*II, *Mbo*I, *Xho*II
*Bgl*I	GCCNNNN↓NGGC	Y		–	
*Bgl*II	A↓GATCT	Y		–	*Bam*HI, *Bcl*I, *Mbo*I, *Xho*II
*Bin*I	GGATC (4/5)[f]		*Alw*I	dam	
*Bse*PI	GCGCGC		*Bss*HII		*Mlu*I
*Bsm*AI	GTCTC				
*Bsm*I	GAATGC (1/−1)[f]	Y			
*Bsp*HI	T↓CATGA	Y	*Rsp*XI	?	*Nco*I
*Bsp*MI	ACCTGC (4/8)[f]	Y			
*Bsp*MII	T↓CCGGA	Y	*Acc*III, *Mro*I	?	*Cfr*10I, *Xma*I
*Bsr*I	ACTGG (1/−1)[f]				
*Bst*EII	G↓GTNACC	Y	*Asp*AI	–	
*Bst*XI	CCANNNNN↓NTGG	Y		?	
*Cau*II	CC↓SGG		*Bcn*I, *Nci*I		
*Cfr*10I	R↓CCGGY	Y			*Bsp*MII, *Xma*I
*Cfr*I	Y↓GGCCR		*Eae*I		*Not*I, *Xma*III
*Cla*I	AT↓CGAT	Y	*Ban*III	dam	*Acy*I, *Asu*II, *Hin*P1I, *Hpa*II, *Mae*II, *Nar*I, *Taq*I
*Cvi*JI	RG↓CY				blunt

*Dde*I	C↓TNAG	Y			
*Dpn*I	G^{m6}A↓TC	Y			blunt
*Dra*II	RG↓GNCCY	Y	*Eco*O109I	dcm	
			*Pss*I (RGGNC↓CY)		
*Dra*III	CACNNN↓GTG	Y			
*Dsa*I	C↓CRYGG				
*Eco*31I	GGTCTC (1/5)^f				
*Eco*47III	AGC↓GCT	Y			blunt
*Eco*57I	CTGAAG (16/14)^f				
*Eco*NI	CCTNN↓NNNAGG			?	
*Eco*RI	G↓AATTC	Y			
*Eco*RII	↓CCWGG	Y		dcm	
			*Apy*I, *Bst*NI, *Mva*I (CC↓WGG)		
*Eco*RV	GAT↓ATC	Y			blunt
*Esp*I	GC↓TNAGC	Y			
*Fin*I	GTCCC			?	
*Fnu*4HI	GC↓NGC	Y			
*Fnu*DII	CG↓CG		*Acc*II, *Bst*UI, *Mvn*I, *Tha*I		blunt
*Fok*I	GGATG (9/13)^f	Y			
*Gdi*II	YGGCCG (−5/−1)^f				
*Gsu*I	CTGGAG (16/14)^f				
*Hae*I	WGG↓CCW			?	blunt
*Hae*II	RGCGC↓Y	Y			*Bbe*I
*Hae*III	GG↓CC	Y	*Bsu*RI, *Pal*I	–	blunt
*Hga*I	GACGC (5/10)^f	Y			
*Hgi*AI	GWGCW↓C	Y			
*Hgi*CI	G↓GYRCC		*Ban*I	?	
*Hgi*EII	ACCNNNNNNGGT			?	
*Hgi*JII	GRGCY↓C		*Ban*II		
*Hha*I	GCG↓C	Y	*Cfo*I		
			*Hin*P1I (G↓CGC)		*Acy*I, *Asu*II, *Cla*I, *Hpa*II, *Mae*II, *Nar*I, *Taq*I
*Hin*dII	GTY↓RAC	Y	*Hin*cII		
*Hin*dIII	A↓AGCTT	Y			
*Hin*fI	G↓ANTC	Y			
*Hpa*I	GTT↓AAC	Y			blunt
*Hpa*II	C↓CGG	Y	*Hap*II, *Msp*I		*Acy*I, *Asu*II, *Cla*I, *Hin*P1I, *Mae*II, *Nar*I, *Taq*I
*Hph*I	GGTGA (8/7)^f	Y		dam	
*Kpn*I	GGTAC↓C	Y		–	
			*Asp*718I (G↓GTACC)		*Spl*I
*Ksp*632I	CTCTTC (1/4)^f	Y	*Ear*I		
*Mae*I	C↓TAG	Y			*Mse*I, *Nde*I, *Vsp*I
*Mae*II	A↓CGT	Y			*Acy*I, *Asu*II, *Cla*I, *Hin*P1I, *Hpa*II, *Nar*I, *Taq*I
*Mae*III	↓GTNAC	Y			
✓ *Mbo*I	↓GATC	Y	*Nde*II, *Sau*3AI	dam	*Bam*HI, *Bcl*I, *Bgl*II, *Xho*II
*Mbo*II	GAAGA (8/7)^f	Y		dam	
*Mlu*I	A↓CGCGT	Y			*Bss*HII

Prototype enzyme	Recognition sequence[a]	Commercially available[b]	Commercial isoschizomers[c]	Methylation sensitivity[d]	Compatible ends[e]
*Mme*I	TCCRAC (20/18)[f]			?	
*Mnl*I	CCTC (7/7)[f]	Y			
*Mse*I	T↓TAA	Y			*Mae*I, *Nde*I, *Vsp*I
*Mst*I	TGC↓GCA		*Aos*I *Fsp*I		blunt
*Nae*I	GCC↓GGC	Y			blunt
*Nar*I	GG↓CGCC	Y	*Nun*II	–	*Acy*I, *Asu*II, *Cla*I, *Hin*P1I, *Hpa*II, *Mae*II, *Taq*I
			*Bbe*I (GGCGC↓C)	?	*Hae*II
*Nco*I	C↓CATGG	Y			*Bsp*HI
*Nde*I	CA↓TATG	Y			*Mae*I, *Mse*I, *Vsp*I
*Nhe*I	G↓CTAGC	Y			*Avr*II, *Spe*I, *Xba*I
*Nla*III	CATG↓	Y			*Nsp*I, *Sph*I
*Nla*IV	GGN↓NCC	Y		?	blunt
*Not*I	GC↓GGCCGC	Y			*Cfr*I, *Xma*III
*Nru*I	TCG↓CGA	Y		dam	blunt
*Nsp*I	RCATG↓Y	Y	*Nsp*HI		*Nla*III, *Sph*I
*Nsp*BII	CMG↓CKG	Y			blunt
*Pfl*MI	CCANNNN↓NTGG	Y		?	
*Ple*I	GAGTC (4/5)[f]	Y			
*Pma*CI	CAC↓GTG				blunt
*Ppu*MI	RG↓GWCCY	Y		?	
*Pst*I	CTGCA↓G	Y			*Nsi*I
*Pvu*I	CGAT↓CG	Y	*Xor*II	–	
*Pvu*II	CAG↓CTG	Y			blunt
*Rsa*I	GT↓AC	Y			blunt
*Rsr*II	CG↓GWCCG	Y			
*Sac*I	GAGCT↓C	Y	*Sst*I		
*Sac*II	CCGC↓GG	Y	*Sst*II		
*Sal*I	G↓TCGAC	Y			*Xho*I
*Sau*I	CC↓TNAGG	Y	*Axy*I, *Bsu*36I, *Cvn*I, *Eco*81I		
*Sca*I	AGT↓ACT	Y			blunt
*Scr*FI	CC↓NGG	Y		dcm	
*Sdu*I	GDGCH↓C	Y	*Bsp*1286I, *Nsp*II	?	
*Sec*I	C↓CNNGG			?	
*Sfa*NI	GCATC (5/9)[f]	Y			
*Sfi*I	GGCCNNNN↓NGGCC	Y		–	
*Sma*I	CCC↓GGG	Y			blunt
			*Xcy*I, *Xma*I (C↓CCGGG)		*Bsp*MII, *Cfr*10I
*Sna*BI	TAC↓GTA	Y			blunt
*Sna*I	GTATAC				
*Spe*I	A↓CTAGT	Y			*Avr*II, *Nhe*I, *Xba*I
*Sph*I	GCATG↓C	Y			*Nla*III, *Nsp*I
*Spl*I	C↓GTACG	Y			*Asp*718I
*Ssp*I	AAT↓ATT	Y			blunt
*Stu*I	AGG↓CCT	Y	*Aat*I	dcm	blunt

*Sty*I	C↓CWWGG	Y	*Eco*T14I		
*Taq*I	T↓CGA	Y	*Tth*HB8I	dam	*Acy*I, *Asu*II, *Cla*I, *Hin*P1I, *Hpa*II, *Mae*II, *Nar*I
*Taq*II	GACCGA (11/9) CACCCA (11/9)^{f,g}			?	
*Tsp*45I	GTSAC				
*Tsp*EI	AATT				
*Tth*111I	GACN↓NNGTC	Y			
*Tth*111II	CAARCA (11/9)^{f}				
*Vsp*I	AT↓TAAT		*Ase*I		*Mae*I, *Mse*I, *Nde*I
*Xba*I	T↓CTAGA	Y		dam	*Avr*II, *Nhe*I, *Spe*I
*Xho*I	C↓TCGAG	Y	*Pae*R7I		*Sal*I
*Xho*II	R↓GATCY	Y	*Bst*YI, *Mfl*I	–	*Bam*HI, *Bcl*I, *Bgl*II, *Mbo*I
*Xma*III	C↓GGCCG	Y	*Eag*I, *Eco*52I		*Cfr*I, *Not*I
*Xmn*I	GAANN↓NNTTC	Y	*Asp*700I		

Enzyme	**Recognition sequence**
(b) *Type I enzymes*	
*Eco*AI	GAGNNNNNNNGTCA
*Eco*BI	TGANNNNNNNNTGCT
*Eco*DI	TTANNNNNNNGTCY
*Eco*DXXI	TCANNNNNNNATTC
*Eco*KI	AACNNNNNNGTGC
*Eco*R124I	GAANNNNNNRTCG
*Eco*R124/3I	GAANNNNNNNRTCG
*Sty*SBI	GAGNNNNNNRTAYG
*Sty*SPI	AACNNNNNNGTRC
*Sty*SQI	AACNNNNNNRTAYG

Enzyme	**Recognition sequence**	**Isoschizomer**
(c) *Type III enzymes*		
*Eco*P15I	CAGCAG	
*Eco*PI	AGACC	
*Hin*fIII	CGAAT	*Hin*eI

See page 5 8 for table notes

Notes (Table 5 1)

Table supplied by R Roberts

[a] Recognition sequences are given using the standard abbreviations (Nomenclature Committee of the International Union of Biochemistry [NC-IUB] 1985) to represent ambiguity R = G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T, H = A or C or T, B = G or T or C, V = G or C or A, D = G or A or T, and N = A or C or G or T Wherever the cleavage site is known, it is indicated by ↓

[b] Consult the manufacturer's instructions for optimal digestion conditions These conditions vary considerably from enzyme to enzyme and may vary between manufacturers for the same enzyme because of differing levels of purity (Y indicates yes)

[c] Occasionally, isoschizomers cleave at a position within the recognition sequence that is different from that of the prototype enzyme Whenever this is the case, the commercially available isoschizomer and its complete recognition sequence are indicated on a separate line

[d] When recognition sites contain sequences that could overlap with *E coli dam* or *dcm* sites, the effects of *dam* or *dcm* methylation are indicated (dam) Inhibited by *dam* methylation, (dcm) inhibited by *dcm* methylation, (–) insensitive to either methylation, (?) effects not documented

[e] Only those enzymes that generate unique termini are considered Note, however, that many enzymes (e g , *Acc*I) can give several different termini, some of which will be compatible with other restriction enzyme termini Usually, only the prototype is listed, i e , unless an isoschizomer cleaves at a different position within the recognition sequence If several isoschizomers give identical termini, then only the most common isoschizomer is listed

[f] Many enzymes cleave at a distance from their recognition sequence, their sites of cleavage are indicated in parentheses For example, *Hga*I GACGC (5/10) indicates cleavage as shown below

```
5' GACGCNNNNN↓        3'
3' CTGCGNNNNNNNNNN↓ 5'
```

In some of these cases, the actual site of cleavage varies slightly, depending on the specific sequence that lies between the recognition and cleavage sites This phenomenon has been noted informally by many investigators, although no systematic study has yet been carried out

[g] *Taq*II differs from other restriction enzymes in recognizing the two distinct sequences shown

TABLE 5.2 Partial List of Restriction Enzymes Ordered by Length of Recognition Sequence

Enzyme	Sequence	Enzyme	Sequence	Enzyme	Sequence	Enzyme	Sequence
*Cvi*JI	RGCY	*Cvi*QI	GTAC	*Ava*I	CYCGRG	*Sph*I	GCATGC
*Mnl*I	CCTC	*Rsa*I	GTAC	*Bsp*1286I	GDGCHC	*Nae*I	GCCGGC
*Alu*I	AGCT	*Taq*I	TCGA	*Aha*II	GRCGYC	*Nhe*I	GCTAGC
*Bsu*FI	CCGG	*Tth*HBI	TCGA	*Aos*II	GRCGYC	*Bam*FI	GGATCC
*Hap*II	CCGG			*Ban*II	GRGCYC	*Bam*HI	GGATCC
*Hpa*II	CCGG	*Scr*FI	CCNGG	*Hgi*JII	GRGCYC	*Bam*KI	GGATCC
*Msp*I	CCGG	*Dde*I	CTNAG	*Acc*I	GTMKAC	*Bam*NI	GGATCC
*Acc*II	CGCG	*Cvi*BI	GANTC	*Hinc*II	GTYRAC	*Bst*I	GGATCC
*Bst*UI	CGCG	*Hha*II	GANTC	*Hgi*AI	GWGCWC	*Bst*1503I	GGATCC
*Bsu*EI	CGCG	*Hin*fI	GANTC	*Cfr*10I	RCCGGY	*Nar*I	GGCGCC
*Fnu*DII	CGCG	*Cfr*13I	GGNCC	*Mfl*I	RGATCY	*Apa*I	GGGCCC
*Tha*I	CGCG	*Sau*96I	GGNCC	*Xho*II	RGATCY	*Asp*718I	GGTACC
*Dpn*I	G^{m6}ATC			*Hae*II	RGCGCY	*Kpn*I	GGTACC
*Nan*II	G^{m6}ATC	*Bcn*I	CCSGG	*Ngo*I	RGCGCY	*Sal*I	GTCGAC
*Nmu*DI	G^{m6}ATC	*Nci*I	CCSGG	*Cfr*I	YGGCCR	*Apa*LI	GTGCAC
*Nmu*EI	G^{m6}ATC	*Aac*I	CCWGG	*Eae*I	YGGCCR	*Hpa*I	GTTAAC
*Bce*243I	GATC	*Aor*I	CCWGG			*Acc*III	TCCGGA
*Bsa*PI	GATC	*Apy*I	CCWGG	*Hin*dIII	AAGCTT	*Bsp*MII	TCCGGA
*Bsp*67I	GATC	*Atu*BI	CCWGG	*Mlu*I	ACGCGT	*Nru*I	TCGCGA
*Bsp*AI	GATC	*Atu*II	CCWGG	*Spe*I	ACTAGT	*Sbo*13I	TCGCGA
*Bsp*PII	GATC	*Bin*SI	CCWGG	*Bgl*II	AGATCT	*Xba*I	TCTAGA
*Bsr*PII	GATC	*Bst*GII	CCWGG	*Stu*I	AGGCCT	*Atu*CI	TGATCA
*Bss*GII	GATC	*Bst*NI	CCWGG	*Ban*III	ATCGAT	*Bcl*I	TGATCA
*Bst*EIII	GATC	*Cfr*5I	CCWGG	*Bsp*XI	ATCGAT	*Bst*GI	TGATCA
*Bst*XII	GATC	*Cfr*11I	CCWGG	*Cla*I	ATCGAT	*Cpe*I	TGATCA
*Cpa*I	GATC	*Eca*II	CCWGG	*Cfr*6I	CAGCTG	*Bal*I	TGGCCA
*Cvi*AI	GATC	*Ecl*II	CCWGG	*Pvu*II	CAGCTG		
*Dpn*II	GATC	*Eco*RII	CCWGG	*Nde*I	CATATG	*Bst*XI	CCAN$_6$TGG
*Fnu*AII	GATC	*Eco*27I	CCWGG	*Nco*I	CCATGG	*Mst*II	CCTNAGG
*Fnu*CI	GATC	*Eco*38I	CCWGG	*Cfr*9I	CCCGGG	*Xmn*I	GAAN$_4$TTC
*Fnu*EI	GATC	*Mph*I	CCWGG	*Sma*I	CCCGGG	*Bgl*I	GCCN$_5$GGC
*Mbo*I	GATC	*Mva*I	CCWGG	*Xma*I	CCCGGG	*Bst*EII	GGTNACC
*Mme*II	GATC	*Taq*XI	CCWGG	*Sac*II	CCGCGG		
*Mno*III	GATC	*Bbv*I	GCAGC	*Pvu*I	CGATCG	*Eco*K	AACN$_6$GTGC
*Mos*I	GATC	*Ava*II	GGWCC	*Rsh*I	CGATCG	*Sty*SPI	AACN$_6$GTRC
*Mth*I	GATC	*Bme*216I	GGWCC	*Xor*II	CGATCG	*Eco*R124	GAAN$_6$RTCG
*Nde*II	GATC	*Eco*47I	GGWCC	*Eag*I	CGGCCG	*Eco*R124/3	GAAN$_7$RTCG
*Nfl*I	GATC	*Sin*I	GGWCC	*Xma*III	CGGCCG	*Eco*A	GAGN$_7$GTCA
*Nla*II	GATC			*Bsu*MI	CTCGAG	*Eco*DXXI	TCAN$_7$ATTC
*Nsi*AI	GATC	*Eco*PI	AGACC	*Pae*R7I	CTCGAG	*Eco*B	TGAN$_8$TGCT
*Nsu*I	GATC	*Mbo*II	GAAGA	*Xho*I	CTCGAG	*Sty*SBI	GAGN$_6$RTAYG
*Pfa*I	GATC	*Hga*I	GACGC	*Pst*I	CTGCAG		
*Sau*3AI	GATC	*Sfa*NI	GCATC	*Sfl*I	CTGCAG	*Csp*I	CGGWCCG
*Sin*MI	GATC	*Bin*I	GGATC	*Eco*RI	GAATTC	*Rsr*II	CGGWCCG
*Hha*I	GCGC	*Fok*I	GGATG	*Rsr*I	GAATTC		
*Hin*PI	GCGC	*Hph*I	GGTGA	*Sso*47I	GAATTC	*Not*I	GCGGCCGC
*Bsu*RI	GGCC	*Bsp*MI	ACCTGC	*Sac*I	GAGCTC	*Sfi*I	GGCCN$_5$GGCC
*Hae*III	GGCC			*Sst*I	GAGCTC		
*Ngo*II	GGCC	*Aqu*I	CYCGRG	*Eco*RV	GATATC		

R - G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T H = A or C or T, B = G or T or C V - G or C or A, D G or A or T
N = A or C or G or T, m^{6}A = 6-methyladenine (Reprinted, with permission from McClelland and Nelson 1988)

LIGATION OF TERMINI CREATED BY RESTRICTION ENZYMES

Compatible Cohesive Termini

If the restriction enzyme cleaves each strand of the substrate DNA on the 5′ side of the axis of dyad symmetry, the resulting staggered break yields fragments of DNA that carry protruding cohesive 5′ termini For example, the enzyme *Eco*RI recognizes the sequence

```
G A A T T C
C T T A A G
```

in double-stranded DNA and cleaves it as follows.

```
              ↓
5′N N N N N G A A T T C N N N N N N3′
3′N N N N N C T T A A G N N N N N N5′
                      ↑
```

The hydrogen bonding of the four base pairs between the sites of cleavage is not favored under the conditions used for digestion. Therefore, the original segment of DNA separates into two fragments

```
5′N N N N N G           pA A T T C N N N N N3′
3′N N N N N C T T A Ap           G N N N N N5′
```

If fragments bearing compatible protruding termini are incubated under conditions that favor the formation of base pairs, they can anneal with one another, and the cleaved phosphodiester bonds can then be resealed with a DNA ligase Because all DNA fragments created by cleavage with *Eco*RI carry the same protruding 5′ termini, they can be joined in novel combinations In fact, *Eco*RI was the first enzyme used to create recombinant DNA molecules (Cohen et al 1973)

If the restriction enzyme cleaves each strand of the DNA on the 3′ side of the axis of dyad symmetry, a staggered break is generated that yields fragments of DNA with protruding cohesive 3′ termini For example, the restriction enzyme *Pst*I recognizes the sequence

```
C T G C A G
G A C G T C
```

in double-stranded DNA and cleaves it as follows

```
                      ↓
5 N N N N N C T G C A G N N N N N N3′
3 N N N N N G A C G T C N N N N N N5′
              ↑
```

```
5′N N N N N C T G C A          pG N N N N N3′
3′N N N N N Gp          A C G T C N N N N N5′
```

Any fragment carrying a protruding terminus generated by cleavage with *Pst*I can be joined to any other fragment carrying the same terminus.

Blunt Ends

Cleavage of both strands of DNA at the axis of dyad symmetry produces blunt-ended fragments For example, the enzyme *Hae*III recognizes the sequence

```
GGCC
CCGG
```

in double-stranded DNA and cleaves it as follows.

```
           ↓
5′ NNNNNNGGCCNNNNNN 3′
3′ NNNNNNCCGGNNNNNN 5′
           ↑
```

to yield fragments that carry blunt ends

```
5′ NNNNNNGG   pCCNNNNNN 3′
3′ NNNNNNCCp   GGNNNNNN 5′
```

Blunt ends generated in this manner can be joined using bacteriophage T4 DNA ligase, although the efficiency of the reaction is somewhat lower than for ligation of cohesive termini Nevertheless, joining of blunt-ended DNA molecules is extremely useful because of its universal application Blunt ends created by restriction enzymes such as *Hae*III can be joined not only to other blunt ends created by *Hae*III or any other restriction enzyme, but also to termini created by the filling of recessed 3′ termini or the removal of protruding 3′ and 5′ termini Blunt-end ligation can also be used to attach synthetic DNA linkers (containing one or more restriction enzyme cleavage sites) to the termini of DNA fragments

Incompatible Cohesive Termini

The joining of DNA fragments with protruding 5′ termini that ordinarily are not compatible (Hung and Wensink 1984) can be accomplished by partial filling of the recessed 3′ termini in controlled reactions using the Klenow fragment of *Escherichia coli* DNA polymerase I In most cases, partial filling also eliminates the ability of compatible termini to pair with each other, thus reducing the frequency of self-joining catalyzed by bacteriophage T4 DNA ligase Hung and Wensink (1984) found that two nonidentical 5′ cohesive termini can be partially filled and then joined efficiently if the resulting single-stranded protruding termini are (1) only one nucleotide in length and complementary, (2) two nucleotides in length and complementary or have a mismatch (e g , dA dC) at one position, or (3) three nucleotides in length and have a mismatch (e g , dT dC) in the central position

An example of the strategy provided by Hung and Wensink (1984) is shown in Figure 5 1 The restriction enzymes *Xba*I and *Hin*dIII generate noncompatible cohesive termini However, if the termini generated by each enzyme are partially filled with the appropriate two nucleotides, the resulting complementary cohesive termini can be joined efficiently by bacteriophage T4 DNA ligase Because the partially filled termini can no longer be joined to themselves, there is no competing self-ligation reaction, and the altered

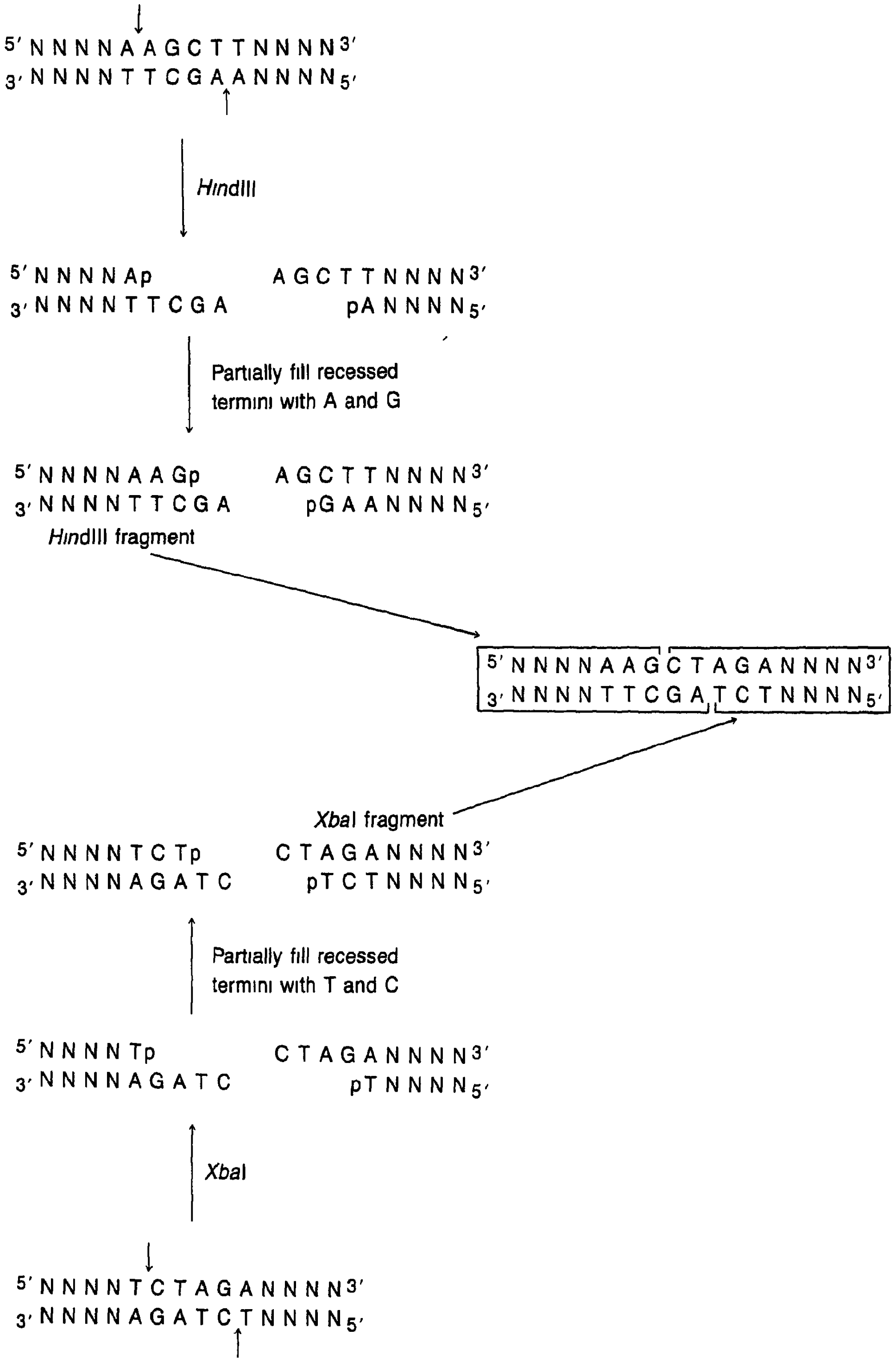


FIGURE 5.1
Ligation of incompatible termini after partial filling. See text for details

termini can therefore be ligated efficiently to one another. Hung and Wensink (1984) also noted that many restriction enzymes generate protruding 5′ cohesive termini whose first nucleotide is complementary to the fourth nucleotide and whose second nucleotide is complementary to the third nucleotide Ligation of mismatched, partially repaired termini allows as many as 70% of the combinations of termini created by different restriction enzymes to be joined.

Two empirical approaches for determining whether any two protruding 5′ termini can be ligated have been reported Hung and Wensink (1984) proposed a simple diagrammatic method in which the sequence of one protruding tetranucleotide 5′ terminus is aligned above the other in opposite polarity If the terminal 5′ nucleotides of each sequence are complementary, then three nucleotides can be added to the recessed termini, and the resulting partially repaired fragments can be ligated via the single remaining complementary nucleotide. If the two terminal 5′ nucleotides of each sequence are complementary, then two nucleotides should be added to the recessed termini If the three terminal nucleotides are complementary, with the exception of the middle nucleotide, then one nucleotide should be added to the recessed termini An alternative method to determine the compatibility of partially filled cohesive termini is to use a cross-index of the type recently published by Korch (1987)

The inability of certain partially filled termini to self-ligate is useful in such applications as the construction of genomic DNA libraries Zabarovsky and Allikmets (1986) inserted genomic DNA partially digested with *Sau*3AI (↓GATC) into a bacteriophage λ vector (EMBL3) that had been cleaved with *Sal*I (G↓TCGAC) The cohesive termini generated by *Sau*3AI and *Sal*I are not normally compatible, but partial repair of the two different tetranucleotide 5′ extensions with two nucleotides generates complementary termini and destroys the ability of the original 5′ termini to self-anneal. Thus, neither the vector nor the genomic DNA fragments can join to themselves, but they can ligate efficiently joined to one another. However, the resulting hybrid joints often cannot be cleaved by any known restriction enzyme, and therefore the cloned fragments cannot be recovered from the vector. This problem can be overcome by cloning into vectors that carry polycloning sites or by choosing combinations of partially repaired termini that, upon ligation, generate a sequence recognized by a known restriction enzyme

ISOSCHIZOMERS

In general, different restriction enzymes recognize different sequences (see Table 5.1). However, there are many examples of enzymes isolated from different sources that cleave within the same target sequences. These are known as isoschizomers. In addition, several enzymes have been discovered that recognize tetranucleotide sequences. In some cases, these tetranucleotides occur within hexanucleotide target sequences of other enzymes. For example, *Mbo*I and *Sau*3AI recognize the sequence

↓
5′ GATC 3′
3′ CTAG 5′
↑

whereas *Bam*HI recognizes

↓
5′ GGATCC 3′
3′ CCTAGG 5′
↑

In each case, however, an identical protruding tetranucleotide terminus is produced. Assuming that restriction enzyme cleavage sites are distributed randomly along the DNA, the tetranucleotide target for *Mbo*I and *Sau*3AI will occur on the average of once every 4^4 (i.e., 256) nucleotides, whereas the hexanucleotide target for *Bam*HI will occur once in every 4^6 (i.e., 4096) nucleotides. DNA fragments generated by complete or partial digestion of eukaryotic DNA with *Mbo*I and *Sau*3AI can therefore be cloned or subcloned in a vector DNA (such as bacteriophage λ or pBR322) that has been cleaved with *Bam*HI (*Note*: Only 1 in every 16 *Mbo*I/*Bam*HI fusions regenerate a *Bam*HI site, and therefore it is not usually possible to recover an *Mbo*I fragment inserted into a *Bam*HI vector by digesting the recombinant with *Bam*HI.)

In a few cases, ligation of fragments generated by one restriction enzyme to those generated by a second enzyme results in hybrids that are recognized by neither of the parental enzymes. For example, when fragments generated by *Sal*I (GTCGAC) are ligated to fragments generated by *Xho*I (CTCGAG), the resulting hybrid target sites are cleaved by neither *Sal*I nor *Xho*I.

*Sal*I fragments *Xho*I fragments
5 G TCGAG 3′
+
3′ CAGCT C 5′
↓
5′ GTCGAG 3′
3′ CAGCTC 5′

DNA METHYLATION

Methylation by Commonly Used Strains of E. coli

Most strains of *E coli* contain two enzymes that methylate DNA—the *dam* methylase and the *dcm* methylase

dam METHYLASE

This enzyme introduces methyl groups at the N^6 position of adenine in the sequence 5′ GATC 3′ (Hattman et al. 1978) The recognition sites of several restriction enzymes (*Pvu*I, *Bam*HI, *Bcl*I, *Bgl*II, *Xho*II, *Mbo*I, and *Sau*3AI) contain this sequence, as do a proportion of the sites recognized by *Cla*I (1 site in 4), *Xba*I (1 site in 16), *Taq*I (1 site in 16), *Mbo*II (1 site in 16), and *Hph*I (1 site in 16) (Table 5 1 indicates the sensitivity of certain restriction enzymes to the *dam* methylase)

The inhibition of *Mbo*I digestion of prokaryotic DNA presents no practical problem because the restriction enzyme *Sau*3AI recognizes exactly the same sequence as *Mbo*I but is unaffected by *dam* methylation (*Note.* Mammalian DNA is not methylated at the N^6 position of adenine, and therefore either *Mbo*I or *Sau*3AI can be used effectively) However, when it is necessary to cleave prokaryotic DNA at every possible site with *Cla*I, *Xba*I, *Taq*I, *Mbo*II, or *Hph*I or to cleave it at all with *Bcl*I, the DNA must be prepared from strains of *E coli* that are *dam*$^-$ (Marinus 1973; Backman 1980, Roberts et al 1980, McClelland 1981)

dcm METHYLASE

This enzyme introduces methyl groups at the C^5 position of the internal cytosine in the sequence 5′ CCAGG 3′ or 5′ CCTGG 3′ (Marinus and Morris 1973, May and Hattman 1975). One enzyme affected by *dcm* methylation is *Eco*RII For most purposes, this problem can be avoided by using *Bst*NI, which recognizes exactly the same sequence as *Eco*RII (although it cuts the DNA at a different location within the sequence). If *Bst*NI cannot be substituted for *Eco*RII, the DNA must be prepared from strains of *E. coli* that are *dcm*$^-$ (Marinus 1973, Backman 1980; Roberts et al 1980) Certain other enzymes may partially overlap the modified *dcm* recognition sequence. (Table 5 1 indicates the sensitivity of certain restriction enzymes to the *dcm* methylase)

METHYLATION-DEPENDENT RESTRICTION SYSTEMS IN *E. coli*

E coli K contains at least three different methylation-dependent restriction systems that recognize different target sequences only when methylated. *mrr* (6-methyladenine [^{m6}A]), *mcrA* (^{m5}CG [^{m5}C = 5-methylcytosine]), and *mcrB* (Pu^{m5}C) (Raleigh and Wilson 1986, Heitman and Model 1987; Raleigh et al. 1988). DNAs that are methylated at such sites are inefficiently cloned into wild-type strains of *E coli* (Whittaker et al 1988) For example, human DNA, which is extensively methylated in vivo at ^{m5}CG, is restricted by *mcrA*. Nonrestricting strains of *E coli* (Raleigh and Wilson 1986; Raleigh et al. 1988) are therefore preferred for transformation and cloning of methylated DNA.

M.*Eco*RI METHYLASE
(*E. coli*)

M.*Eco*RI methylase catalyzes the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to the adenines marked * in the *Eco*RI recognition sequence

$\mathsf{G\ A\ \overset{*}{A}\ T\ T\ C}$

$\mathsf{C\ T\ T\ \underset{*}{A}\ A\ G}$

The modification of adenine to 6-methylaminopurine protects the DNA from cleavage by *Eco*RI (Greene et al 1975)

Modification of Restriction Sites by DNA Methylation

For many of the type II restriction enzymes, a corresponding methylase has been isolated that modifies the cognate recognition sequence and renders it resistant to cleavage. These methylases, a number of which are available from commercial suppliers, are useful in a number of tasks in molecular cloning For example, in the construction of genomic DNA libraries (Maniatis et al. 1978), random fragments of genomic DNA generated by partial cleavage with the restriction enzymes *Alu*I and *Hae*III can be treated with M *Eco*RI methylase prior to the addition of synthetic *Eco*RI linkers When the linkers attached to genomic DNA are subsequently digested with *Eco*RI, the natural restriction sites within the genomic DNA are protected from cleavage The same strategy may be used to spare natural restriction sites when preparing double-stranded cDNA for cloning

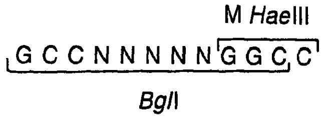
Methylases can also be used to alter the apparent cleavage specificity of certain restriction enzymes (Nelson et al. 1984, Nelson and Schildkraut 1987) These alterations are accomplished in vitro by methylation of a subset of the sequences recognized by certain restriction enzymes Only the methylated subsets will be resistant to cleavage. For example, the restriction enzyme *Hin*cII recognizes the degenerate sequence GTPyPuAC and will therefore cleave the four sequences·

GTCGAC
GTCAAC
GTTGAC
GTTAAC

The M.*Taq*I methylase recognizes only the sequence TCGA and methylates the adenine residue (McClelland 1981) The subset of *Hin*cII recognition sequences that contains the internal sequence TCGA will therefore be resistant to cleavage after methylation by M *Taq*I, whereas the other three recognition sequences will remain sensitive to *Hin*cII

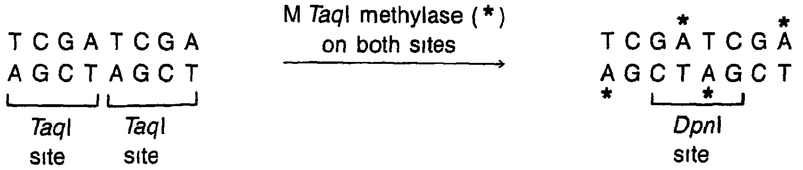
A second class of overlapping methylation and restriction sites occurs at the boundaries of the recognition sequences of a restriction enzyme and a methylase. For example, a *Bam*HI site (GGATCC) that happens to be preceded by CC or followed by GG partially overlaps a site (CCGG) that can be methylated by the enzyme M *Msp*I. Because M.*Msp*I methylates the 5′ cytosine of its recognition sequence (^{m5}CCGG), the *Bam*HI site becomes methylated at an internal cytosine residue (GGAT^{m5}CCGG) and, therefore, is resistant to cleavage by *Bam*HI.

Another example is provided by *Bgl*I and M *Hae*III.



As in the cases of *Hinc*II/M *Taq*I and *Bam*HI/M.*Msp*I, the methylation blocks the cleavage of a previously existing restriction site.

In a final example, certain adenine methylases can be used in conjunction with the methylation-dependent restriction enzyme *Dpn*I to produce highly specific cleavages at sequences 8–12 bp in length (McClelland et al. 1984; McClelland and Nelson 1987; Weil and McClelland 1989).



This example differs from those above in that this strategy *creates* a site in DNA that otherwise would not be cleaved by *Dpn*I. This general strategy of using specific methylases in conjunction with restriction enzymes has produced over 60 new cleavage specificities and many more are possible (for references, see McClelland and Nelson 1988).

Table 5.3 provides a compilation of the sensitivities of restriction enzymes to site-specific modifications commonly found in bacterial and eukaryotic DNAs and in viruses.

Several pairs of isoschizomers differ in their sensitivity to site-specific methylation (see Table 5.4). Such endonuclease pairs are useful for studying the level and distribution of site-specific methylation in cellular DNA, for example, ^{m5}CG in mammals, ^{m5}CG and ^{m5}CNG in plants, or G^{m6}ATC in enterobacteria (Waalwijk and Flavell 1978; McClelland 1983; Bird et al. 1985).

Sensitivity to site-specific DNA methylation is clearly not limited to restriction enzymes but is a property of DNA-binding proteins in general (see Sternberg 1985; Wang et al. 1986). ^{m4}C, ^{m5}C, ^{hm5}C, and ^{m6}A site-specific modification at "noncanonical" sites will block several type II methylases. The data are summarized in Table 5.5.

TABLE 5.3 Methylation Sensitivity of Restriction Enzymes

Restriction enzyme	Recognition sequence	Sites cut	Sites not cut	Comments
*Aac*I	CCWGG	C^{m5}CWGG	?	
*Aat*I	AGGCCT	?	AGG^{m5}CCT AGGC^{m5}CT	
*Acc*I	GTMKAC	?	GTMK^{m6}AC GTMKA^{m5}C	*Acc*I nicking occurs slowly in the unmethylated strand of the hemimethylated sequence GTMKA^{m5}C
*Acc*II	CGCG	?	^{m5}CGCG	
*Acc*III	TCCGGA	T^{m5}CCGGA TC^{m5}CGGA	TCCGG^{m6}A	
*Afl*I	GGWCC	GGWC^{m5}C	?	
*Aha*II	GRCGYC	?	GR^{m5}CGYC GRCGY^{m5}C	*Aha*II will cut GRCGCC *faster* if these sites are methylated at GRCG^{m5}CC but will not cut GRCGY^{m5}C sites
*Alu*I	AGCT	?	AG^{m5}CT$^{\#}$ AG^{m4}CT AG^{hm5}CT ^{m6}AGCT	
*Aos*II	GRCGYC	?	GR^{m5}CGYC	
*Apa*I	GGGCCC	?	GGG^{m5}CCC$^{\#}$ GGGCC^{m5}C	
*Apa*LI	GTGCAC	GTGC^{m6}AC	?	
*Apy*I	CCWGG	C^{m5}CWGG	^{m5}CCWGG	
*Aqu*I	CYCGRG	?	^{m5}CYCGRG$^{\#}$	
*Asp*718I	GGTACC	?	GGTAC^{m5}C	
*Asu*II	TTCGAA	TT^{m5}CGAA	?	
*Atu*CI	TGATCA	?	TG^{m6}ATCA	
*Ava*I	CYCGRG	C^{m5}CCGGG	^{m5}CYCGRG CY^{m5}CGRG CTCG^{m6}AG	*Ava*I nicking occurs slowly in the unmethylated strand of the hemimethylated sequence CTCG^{m6}AG/CTCGAG
*Ava*II	GGWCC	?	GGWC^{m5}C GGW^{m5}CC GGW^{hm5}C^{hm5}C	
*Bal*I	TGGCCA	?	TGG^{m5}CCA$^{\#}$ TGGC^{m5}CA	*Bal*I sites overlapping *dcm* sites (TGGC^{m5}CAGG) are cleaved 50-fold slower than unmethylated sites
*Bam*FI	GGATCC	GG^{m6}ATCC	?	
*Bam*HI	GGATCC	GGATC^{m5}C GG^{m6}ATCC GG^{m6}ATC^{m5}C	GGAT^{m4}CC$^{\#}$ GGAT^{m5}CC GGAT^{hm5}C^{hm5}C	
*Bam*KI	GGATCC	GG^{m6}ATCC	?	
*Ban*I	GGYRCC	GG^{m5}CGCC	?	*Ban*I gives various rate effects when the recognition sequence is ^{m5}C-methylated at different sites
*Ban*II	GRGCYC	?	GRG^{m5}CYC	
*Ban*III	ATCGAT	?	ATCG^{m6}AT	
*Bbv*I	GCAGC	?	G^{m5}CAGC$^{\#}$	
*Bcl*I	TGATCA	TGAT^{m5}CA	TG^{m6}ATCA TGAT^{hm5}CA	M*Bcl*I may be ^{m6}A-specific
*Bcn*I	CCSGG	^{m5}CCSGG	C^{m4}CSGG$^{\#}$	

*Bgl*I	GCCN$_5$GGC	GC^{m5}CN$_5$GGC	GCCN$_5$GG^{m5}C G^{m5}CCN$_5$GGC	*Bgl*I cleavage at certain hemimethylated ^{m5}C sites varies (overlapping M.*Msp*I-*Bgl*I and M.*Hpa*II-*Bgl*I sites) ^{m5}C-bimethylated M.*Hae*III-*Bgl*I sites are completely refractory to *Bgl*I
*Bgl*II	AGATCT	AG^{m6}ATCT	AGAT^{m5}CT AGAT^{hm5}CT	M.*Bgl*II may be ^{m4}C-specific
*Bin*I	GGATC	?	GG^{m6}ATC	
*Bme*216I	GGWCC	?	GGWC^{m5}C	
*Bsp*1286I	GDGCHC	?	GDG^{m5}CHC	
*Bsp*MI	ACCTGC	?	ACCTG^{m5}C	
*Bsp*MII	TCCGGA	TCCGG^{m6}A	TC^{m5}CGGA T^{m5}CCGGA	
*Bsp*XI	ATCGAT	?	ATCG^{m6}AT	
*Bst*EII	GGTNACC	GGTNA^{m5}C^{m5}C	GGTNA^{hm5}C^{hm5}C	*Bst*EII cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA
*Bst*EIII	GATC	?	G^{m6}ATC	
*Bst*GI	TGATCA	?	TG^{m6}ATCA	
*Bst*I	GGATCC	GG^{m6}ATCC	GGAT^{m5}CC	
*Bst*NI	CCWGG	C^{m5}CWGG ^{m5}CCWGG ^{m5}C^{m5}CWGG	^{hm5}C^{hm5}CWGG	*Bst*NI cuts C^{m5}CWGG, ^{m5}CCWGG, and ^{m5}C^{m5}CWGG M.*Bst*NI may be a ^{m4}C-methylase *Bst*NI isoschizomers that are insensitive to C^{m5}CWGG include *Aor*I, *Apy*I, *Mva*I, and *Taq*XI
*Bst*UI	CGCG	?	^{m5}CG^{m5}CG	
*Bst*XI	CCAN$_6$TGG	?	^{m5}CCAN$_6$TGG CC^{m6}AN$_6$TGG	
*Bsu*EI	CGCG	?	^{m5}CGCG*	
*Bsu*FI	CCGG	?	^{m5}CCGG*	
*Bsu*MI	CTCGAG	?	CT^{m5}CGAG*	
*Bsu*RI	GGCC	?	GG^{m5}CC*	*Bsu*RI nicking occurs in the unmethylated strand of the hemimethylated sequence GG^{m5}CC/GGCC
*Cfo*I	GCGC	?	G^{hm5}CG^{hm5}C G^{m5}CGC	
*Cfr*I	YGGCCR	?	YGG^{m5}CCR*	
*Cfr*6I	CAGCTG	?	CAG^{m4}CTG* CAG^{m5}CTG	
*Cfr*9I	CCCGGG	C^{m4}CCGGG CC^{m5}CGGG	^{m4}CCCGGG C^{m4}CCGGG* CC^{m4}CGGG ^{m5}CCCGGG	See Butkus et al (1987) for rate effects
*Cfr*10I	RCCGGY	?	R^{m5}CCGGY*	
*Cfr*13I	GGNCC	?	GGN^{m5}CC*	
*Cla*I	ATCGAT	?	ATCG^{m6}AT* ^{m6}ATCGAT AT^{m5}CGAT	
*Cpe*I	TGATCA	?	TG^{m6}ATCA	
*Csp*I	CGGWCCG	?	CGGW^{m5}CCG ^{m5}CGGWC^{m5}CG	
*Cvi*AI	GATC	?	G^{m6}ATC	
*Cvi*BI	GANTC	?	G^{m6}ANTC*	
*Cvi*JI	RGCY	?	RG^{m5}CY*	
*Cvi*QI	GTAC	GTA^{m5}C	GT^{m6}AC*	
*Dde*I	CTNAG	?	^{m5}CTNAG ^{hm5}CTNAG	

Restriction enzyme	Recognition sequence	Sites cut	Sites not cut	Comments
*Dpn*I	G^{m6}ATC	G^{m6}ATC G^{m6}AT^{m5}C	GATC GAT^{m5}C	*Dpn*I requires adenine methylation on both DNA strands Isoschizomers of *Dpn*I include *Cfu*I, *Nan*II, *Nmu*DI, *Nmu*EI, and *Nsu*DI *Dpn*I cuts *dam*-modified bacteriophage XP12 DNA
*Dra*II	RGGNCCY	?	RGGNC^{m5}CY	
*Eae*I	YGGCCR	?	YGG^{m5}CCR* YGGC^{m5}CR	
*Eag*I	CGGCCG	?	CGG^{m5}CCG ^{m5}CGGC^{m5}CG	
*Eco*A	GAGN$_7$GTCA	?	G^{m6}AGN$_7$G^{m6}TCA*	*Eco*A is a type I restriction enzyme ^{m6}T represents a 6-methyladenine in the complementary strand
*Eco*B	TGAN$_8$TGCT	?	TG^{m6}AN$_8$^{m6}TGCT	*Eco*B is a type I restriction enzyme ^{m6}T represents a 6-methyladenine in the complementary strand
*Eco*DXXI	TCAN$_7$ATTC	?	TCAN$_7$^{m6}AT^{m6}TC	*Eco*DXXI is a type I restriction enzyme ^{m6}T represents a 6-methyladenine in the complementary strand
*Eco*47I	GGWCC	?	GGWC^{m5}C	
*Eco*K	AACN$_6$GTGC	?	A^{m6}ACN$_6$G^{m6}TGC*	*Eco*K is a type I restriction enzyme ^{m6}T represents a 6-methyladenine in the complementary strand
*Eco*O109I	RGGNCCY	?	RGGNC^{m5}CY	
*Eco*PI	AGACC	AGA^{hm5}C^{hm5}C	AG^{m6}ACC*	*Eco*PI is a type III restriction enzyme
*Eco*R124	GAAN$_6$RTCG	?	^{m6}A	*Eco*R124 is a type I restriction enzyme
*Eco*R124/3	GAAN$_7$RTCG	?	^{m6}A	*Eco*R124/3 is a type I restriction enzyme
*Eco*RI	GAATTC	GAATT^{hm5}C	GA^{m6}ATTC* G^{m6}AATTC GAATT^{m5}C	*Eco*RI cannot cut hemimethylated G^{m6}AATTC/GAATTC sites Bimethylated GA^{m6}ATTC/GA^{m6}ATTC sites are not cut by *Eco*RI or *Rsr*I *Eco*RI shows a reduced rate of cleavage at hemimethylated GAATT^{m5}C/GAATTC and does not cut an oligonucleotide that contains GAATT^{m5}C in both strands
*Eco*RII	CCWGG	^{m5}CCWGG	C^{m5}CWGG* C^{m4}CWGG ^{hm5}C^{hm5}CWGG CC^{m6}AGG	*Eco*RII isoschizomers that are sensitive to C^{m5}CWGG include *Atu*BI, *Atu*II, *Bin*SI, *Bst*GII, *Cfr*5I, *Cfr*11I, *Eca*II, *Ecl*II, *Eco*27I, *Eco*38I, and *Mph*I
*Eco*RV	GATATC	GATAT^{m5}C	G^{m6}ATATC*	*Eco*RV cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA
*Fnu*DII	CGCG	?	^{m5}CGCG CG^{m5}CG	
*Fnu*EI	GATC	G^{m6}ATC	?	
*Fnu*4HI	GCNGC	?	G^{m5}CNGC GCNG^{m5}C	
*Fok*I	GGATG (5′ CATCC 3′)	CATC^{m5}C CAT^{m5}CC	GG^{m6}ATG	*Fok*I cuts about two- to fourfold more slowly at CATC^{m5}C than at unmodified sites
*Hae*II	RGCGCY	?	RG^{m5}CGCY RG^{hm5}CG^{hm5}CY	*Hae*II gives various rate effects when the recognition sequence is ^{m5}C-methylated at different sites M.*Hae*II may be ^{m5}C-specific
*Hae*III	GGCC	GGC^{m5}C	GG^{m5}CC* GG^{hm5}C^{hm5}C	*Hae*III nicking occurs in the unmethylated strand of the hemimethylated sequence GG^{m5}CC/GGCC
*Hap*II	CCGG	?	C^{m5}CGG	
*Hga*I	GACGC	?	GACG^{m5}C	M.*Hga*I may be ^{m4}C- or ^{m6}A-specific
*Hgi*AI	GWGCWC	?	GWG^{m5}CWC	
*Hgi*JII	GRGCYC	?	GRGCY^{m5}C	

*Hha*I	GCGC	?	G^{m5}CGC$^{\#}$ GCG^{m5}C G^{hm5}CG^{hm5}C	
*Hha*II	GANTC	?	G^{m6}ANTC$^{\#}$	
*Hinc*II	GTYRAC	GTYRA^{m5}C	GTYR^{m6}AC GTYRA^{hm5}C	
*Hind*II	GTYRAC	?	GTYR^{m6}AC$^{\#}$	
*Hind*III	AAGCTT	?	^{m6}AAGCTT$^{\#}$ AAG^{m5}CTT AAG^{hm5}CTT	
*Hinf*I	GANTC	GANT^{m5}C	G^{m6}ANTC GANT^{hm5}C	*Hinf*I cuts GANT^{m5}C, however, detectable rate differences are observed between unmethylated, hemimethylated (GANT^{m5}C/GANTC), and bimethylated (GANT^{m5}C/GANT^{m5}C) target sequences *Hinf*I does cut bacteriophage XP12 DNA, although at a reduced rate *Hinf*I cuts unmethylated GANTC faster than hemimethylated GANT^{m5}C/GANTC, which is cut faster than GANT^{m5}C/GANT^{m5}C However, the rate difference between unmethylated and fully methylated *Hinf*I sites is only about tenfold
*Hin*PI	GCGC	?	G^{m5}CGC	
*Hpa*I	GTTAAC	GTTAA^{m5}C	GTTA^{m6}AC$^{\#}$ GTTAA^{hm5}C	
*Hpa*II	CCGG	?	C^{m5}CGG$^{\#}$ ^{m5}CCGG ^{m4}CCGG C^{m4}CGG ^{hm5}C^{hm5}CGG	*Hpa*II nicking occurs in the unmethylated strand of the hemimethylated sequence ^{m5}CCGG/CCGG See Butkus et al (1987) for rate effects
*Hph*I	GGTGA (5' TCACC 3')	?	T^{m5}CACC$^{\#}$ GGTG^{m6}A	
*Kpn*I	GGTACC	GGTA^{m4}CC GGTAC^{m5}C GGTA^{m4}C^{m4}C	?	*Kpn*I sensitivity to hemimethylated GGTA^{m5}CC/GGTACC and GGTAC^{m5}C/GGTACC sites is in dispute It is possible that rate effects are observed at certain ^{m5}C-methylated *Kpn*I sites, especially at low enzyme to substrate ratios *Kpn*I cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA
*Mae*II	ACGT	?	A^{m5}CGT	*Mae*II nicks slowly in the unmethylated strand of hemimethylated A^{m5}CGT/ACGT
*Mbo*I	GATC	GAT^{m5}C	G^{m6}ATC GAT^{hm5}C	*Mbo*I isoschizomers that are sensitive to G^{m6}ATC include *Bsa*PI, *Bss*GII, *Bst*EIII, *Bst*XII, *Cpa*I, *Dpn*II, *Fnu*AII, *Fnu*CI, *Mme*II, *Mno*III, *Mos*I, *Nde*II, *Nfl*I, *Nla*II, *Nsu*I, and *Sin*MI
*Mbo*II	GAAGA (5' TCTTC 3')	T^{m4}CTT^{m5}C	GAAG^{m6}A$^{\#}$	*Mbo*II cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA, although certain hemimethylated ^{m5}C-containing substrates are reported not to be cut
*Mfl*I	RGATCY	?	RG^{m6}ATCY RGAT^{m5}CY RGAT^{m4}CY	*Mfl*I cuts more slowly at ^{m6}AGATCY sites
*Mlu*I	ACGCGT	^{m6}ACGCGT	A^{m5}CGCGT	
*Mme*II	GATC	?	G^{m6}ATC	
*Mnl*I	CCTC	?	^{m5}CCTC ^{m5}C^{m5}CT^{m5}C	M *Mnl*I may be a ^{m5}C- or ^{m6}A-specific methylase

Restriction enzyme	Recognition sequence	Sites cut	Sites not cut	Comments
*Mph*I	CCWGG	?	C^{m5}CWGG	
*Msp*I	CCGG	C^{m5}CGG ^{m4}CCGG C^{m4}CGG	^{m5}CCGG$^{\#}$ ^{hm5}C^{hm5}CGG	*Msp*I cuts the unmethylated and methylated strands of C^{m5}CGG/CCGG and C^{m4}CGG/CCGG duplexes *Msp*I cuts very slowly at GGC^{m5}CGG An M.*Msp*I clone methylates ^{m5}CCGG However, there is a report that *Moraxella* species chromosomal DNA is methylated at ^{m5}C^{m5}CGG
*Mst*II	CCTNAGG	^{m5}CCTNAGG	?	
*Mva*I	CCWGG	C^{m5}CWGG ^{m5}CCWGG	C^{m4}CWGG$^{\#}$ CC^{m6}AGG ^{m4}CCWGG	*Mva*I nicking occurs in the unmethylated strand of the hemimethylated sequence C^{m4}CWGG/CCWGG
*Nae*I	GCCGGC	?	G^{m5}CCGGC GC^{m5}CGGC GCCGG^{m5}C	M.*Nae*I may be ^{m5}C-specific
*Nan*II	G^{m6}ATC	G^{m6}ATC G^{m6}AT^{m5}C	GATC GAT^{m5}C	*Nan*II requires adenine methylation on both DNA strands *Nan*II cuts *dam*-modified bacteriophage XP12 DNA *Nan*II is an isoschizomer of *Dpn*I
*Nar*I	GGCGCC	GGCGC^{m5}C	GG^{m5}CGCC	
*Nci*I	CCSGG	^{m5}CCSGG	C^{m5}CSGG C^{m4}CSGG	*Nci*I may cut ^{m5}C^{m5}CSGG methylated DNA. It is possible that the second methylation negates the effect of C^{m5}CSGG
*Nco*I	CCATGG	?	^{m5}CCATGG	*Nco*I is blocked by M *Sec*I (CCNNGG)
*Nde*I	CATATG	^{m5}CATATG	^{m6}A	*Nde*I cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA
*Nde*II	GATC	GAT^{m5}C	G^{m6}ATC	*Nde*II cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA
*Ngo*I	RGCGCY	?	RG^{m5}CGCY	
*Ngo*II	GGCC	?	GG^{m5}CC	
*Nhe*I	GCTAGC	?	GCTAG^{m5}C	
*Nmu*DI	G^{m6}ATC	G^{m6}ATC	GATC	*Nmu*DI requires adenine methylation on both DNA strands *Nmu*DI is an isoschizomer of *Dpn*I
*Nmu*EI	G^{m6}ATC	G^{m6}ATC	GATC	*Nmu*E1 requires adenine methylation on both DNA strands *Nmu*EI is an isoschizomer of *Dpn*I
*Not*I	GCGGCCGC	GCGGCCG^{m5}C	GCGG^{m5}CCGC GCGGC^{m5}CGC	
*Nru*I	TCGCGA	?	TCGCG^{m6}A	
*Pae*R7I	CTCGAG	?	CTCG^{m6}AG$^{\#}$	
*Pfa*I	GATC	G^{m6}ATC	?	
*Pst*I	CTGCAG	?	CTGC^{m6}AG$^{\#}$ ^{m5}CTGCAG	
*Pvu*I	CGATCG	CG^{m6}ATCG	CGAT^{m4}CG CGAT^{m5}CG	M *Pvu*I may be ^{m5}C-specific
*Pvu*II	CAGCTG	?	CAG^{m4}CTG$^{\#}$ CAG^{m5}CTG	
*Rsa*I	GTAC	GTA^{m5}C	?	*Rsa*I cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA M *Rsa*I may be ^{m4}C- or ^{m6}A-specific
*Rsh*I	CGATCG	CG^{m6}ATCG	?	
*Rsr*I	GAATTC	?	G^{m6}AATTC GA^{m6}ATTC	*Rsr*I cannot cut hemimethylated G^{m6}AATTC/GAATTC or bimethylated GA^{m6}ATTC/GA^{m6}ATTC sites
*Rsr*II	CGGWCCG	?	CGGW^{m5}CCG ^{m5}CGGWC^{m5}CG	
*Sac*I	GAGCTC	G^{m6}AGCTC	GAG^{m5}CTC	
*Sac*II	CCGCGG	?	^{m5}CCGCGG	

*Sal*I	GTCGAC	?	GTCG^{m6}AC GT^{m5}CGAC	
*Sau*3AI	GATC	G^{m6}ATC	GAT^{m5}C GAT^{m4}C GAT^{hm5}C	*Sau*3AI nicking occurs in the unmethylated strand of the hemimethylated sequence GAT^{m5}C/GATC *Sau*3AI cuts at a reduced rate at ^{m6}AGATC *Sau*3AI isoschizomers that are insensitive to G^{m6}ATC include *Bce*243I, *Bsp*67I, *Bsp*AI, *Bsp*PII, *Bsr*PII, *Fnu*EI, *Mth*I, *Nsi*AI, and *Pfa*I
*Sau*96I	GGNCC	?	GGN^{m5}CC GGNC^{m5}C GGN^{hm5}C^{hm5}C	
*Sbo*13I	TCGCGA	TCGCG^{m6}A	?	
*Scr*FI	CCNGG	^{m5}CCNGG	C^{m5}CNGG	
*Sfa*NI	GCATC (5′ GATGC 3′)	GATG^{m5}C	G^{m6}ATGC	
*Sfi*I	GGCCN$_5$GGCC	GG^{m5}CCN$_5$GG^{m5}CC GGCCN$_5$GGC^{m5}C	?	
*Sfl*I	CTGCAG	?	CTGC^{m6}AG	
*Sin*I	GGWCC	?	GGW^{m5}CC	
*Sma*I	CCCGGG	C^{m5}CCGGG	^{m5}CCCGGG CC^{m5}CGGG ^{m4}CCCGGG C^{m4}CCGGG CC^{m4}CGGG	*Sma*I nicking occurs in the unmethylated strand of the hemimethylated sequence CC^{m5}CGGG/CCCGGG M*Sma*I may be ^{m5}C-specific *Sma*I may cut C^{m5}C^{m5}CGGG methylated DNA It is possible that the second methylation negates the effect of CC^{m5}CGGG There are conflicting results regarding *Sma*I ^{m5}CCCGGG is not cut when modified by M.*Aqu*I methylase or at overlapping M*Hae*III-*Sma*I sites (GG^{m5}CCCGGG), other investigators have reported that *Sma*I cuts at a reduced rate at hemimethylated ^{m5}CCCGGG/CCCGGG sites
*Spe*I	ACTAGT	?	^{m6}ACTAGT	
*Sph*I	GCATGC	GCATG^{m5}C G^{hm5}CATG^{hm5}C	?	
*Sso*47I	GAATTC	?	G^{m6}AATTC*	
*Ssu*II	CCNGG	?	C^{m5}CNGG ^{m5}CCNGG	
*Sst*I	GAGCTC	?	GAG^{m5}CTC GAG^{hm5}CT^{hm5}C	
*Stu*I	AGGCCT	?	AGG^{m5}CCT AGGC^{m5}CT	
*Sty*SBI	GAGN$_6$RTAYG	?	G^{m6}AGN$_6$R^{m6}TAYG*	*Sty*SBI is a type I restriction enzyme ^{m6}T represents a 6-methyladenine in the complementary strand
*Sty*SPI	AACN$_6$GTRC	?	A^{m6}ACN$_6$G^{m6}TRC*	*Sty*SPI is a type I restriction enzyme ^{m6}T represents a 6-methyladenine in the complementary strand
*Taq*I	TCGA	T^{m6}CGA T^{hm5}CGA	TCG^{m6}A*	*Taq*I cuts very slowly at T^{hm5}CGA *Taq*I cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA
*Taq*XI	CCWGG	^{m5}CCWGG C^{m5}CWGG	?	
*Tha*I	CGCG	?	^{m5}CGCG ^{hm5}CG^{hm5}CG	
*Tth*HBI	TCGA	T^{m6}CGA	TCG^{m6}A*	
*Xba*I	TCTAGA	?	T^{m5}CTAGA TCTAG^{m6}A T^{hm5}CTAGA	
*Xho*I	CTCGAG	?	CT^{m5}CGAG CTCG^{m6}AG ^{m5}CTCGAG	M.*Xho*I may be ^{m4}C- or ^{m6}A-specific

Restriction enzyme	Recognition sequence	Sites cut	Sites not cut	Comments
*Xho*II	RGATCY	RG^{m4}ATCY	RGAT^{m5}CY	*Xho*II nicking occurs slowly in the unmethylated strand of the hemimethylated sequence RGAT^{m5}CY/RGATCY
*Xma*I	CCCGGG	CC^{m5}CGGG	^{m4}CCCGGG C^{m4}CCGGG CC^{m4}CGGG ^{m5}CCCGGG	*Xma*I is claimed not to cut CC^{m5}CGGG in one report See Butkus et al (1987) for rate effects
*Xma*III	CGGCCG	?	CGG^{m5}CCG	
*Xmn*I	GAAN$_4$TTC	GA^{m6}AN$_4$TTC	G^{m6}AAN$_4$TTC GAAN$_4$TT^{m5}C	*Xmn*I cuts the fully ^{m5}C-substituted bacteriophage XP12 DNA *Xmn*I cuts slowly at some sites in DNA methylated on *both* strands at GAAN$_4$TT^{m5}C
*Xor*II	CGATCG	CG^{m6}ATCG	CGAT^{m5}CG ^{hm5}CGAT^{hm5}CG	

[#] denotes canonical modification methylase specificity R = G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T, H = A or C or T, B = G or T or C, V = G or C or A, D = G or A or T, N = A or C or G or T, ^{m4}C = 4-methylcytosine, ^{m5}C = 5-methylcytosine, ^{hm5}C = 5-hydroxymethylcytosine, and ^{m6}A = 6-methyladenine For references, see McClelland and Nelson (1988) (Reprinted, with permission, from McClelland and Nelson 1988 with modifications)

TABLE 5.4 Isoschizomer Pairs That Differ in Their Sensitivity to Sequence-specific Methylation

	Isoschizomer pairs[a]	
Methylated sequence[b]	cut by	not cut by
T^{m5}CCGGA	*Acc*III	*Bsp*MII
TC^{m5}CGGA	*Acc*III	*Bsp*MII
GGWC^{m5}C	*Afl*I	*Ava*II (*Eco*47I)
TCCGG^{m6}A	*Bsp*MII	*Acc*III
C^{m5}CWGG	*Bst*NI (*Mva*I)	*Eco*RII
GGTAC^{m5}C	*Kpn*I	*Asp*718I
C^{m5}CGG	*Msp*I	*Hpa*II (*Hap*II)
C^{m4}CGG	*Msp*I	*Hpa*II
G^{m6}ATC	*Sau*3AI (*Fnu*EI)	*Mbo*I (*Nde*II)
TCGCG^{m6}A	*Sbo*13I	*Nru*I
RG^{m6}ATCY	*Xho*II	*Mfl*I
CC^{m5}CGGG	*Xma*I (*Cfr*9I)	*Sma*I

In each row, the first column lists a methylated sequence, the second column lists an isoschizomer that cuts this sequence, and the third column lists an isoschizomer that does not cut this sequence For references, see McClelland and Nelson (1988) (Reprinted, with permission, from McClelland and Nelson 1988)

[a] An enzyme is classified as insensitive to methylation if it cuts the methylated sequence at a rate that is at least one tenth the rate at which it cuts the unmethylated sequence An enzyme is classified as sensitive to methylation if it is inhibited at least 20-fold by methylation relative to the unmethylated sequence

[b] Sequences are in 5′→3′ order R = G or A, Y = C or T, W = A or T, ^{m4}C = 4-methylcytosine, ^{m5}C = 5-methylcytosine and ^{m6}A = 6-methyladenine

TABLE 5.5 Methylation Sensitivity of Type II DNA Methylases

Methylase (specificity)[a]	Not blocked by prior modification at[b]	Blocked by prior modification at[b]
M.*Alu*I (AG^{m5}CT)		AG^{m4}CT
M *Bam*HI (GGAT^{m4}CC)	GG^{m6}ATCC	GGATC^{m5}C
M *Cfr*6I (CAG^{m4}CTG)		CAG^{m5}CTG
M *Cla*I (ATCG^{m6}AT)	^{m6}ATCGAT AT^{m5}CGAT	
M *Cvi*BIII (TCG^{m6}A)	T^{m5}CGA	
M *Eco*RI (GA^{m6}ATTC)	GAATT^{m5}C	G^{m6}AATTC
M *Eco*RII (C^{m5}CWGG)		C^{m4}CWGG
E coli dam (G^{m6}ATC)	GAT^{m5}C[c] GAT^{hm5}C	
M *Fok*IA (GG^{m6}ATG)	CATC^{m5}C	CAT^{m5}CC
M *Hha*I (G^{m5}CGC)	GCG^{m5}C	
M *Hha*II (G^{m6}ANTC)	GANT^{m5}C	
M *Hpa*II (C^{m5}CGG)		^{m5}CCGG
M *Hph*I (T^{m5}CACC)	GGTG^{m6}A	
M *Mbo*I (G^{m6}ATC)	GAT^{m5}C	
M *Mbo*II (GAAG^{m6}A)	T^{m5}CTT^{m5}C	
M.*Msp*I (^{m5}CCGG)		C^{m5}CGG
M.*Mva*I (C^{m4}CWGG)	C^{m5}CWGG	
M *Pvu*II (CAG^{m4}CTG)		CAG^{m5}CTG
M T2 *dam* (G^{m6}ATC)	GAT^{hm5}C	
M T4 *dam* (G^{m6}ATC)	GAT^{hm5}C	
M *Taq*I (TCG^{m6}A)	T^{m5}CGA	

(Reprinted, with permission, from McClelland and Nelson 1988) For references, see McClelland and Nelson (1988)

[a] Sequences are in 5′→3′ order W = A or T, N = A or C or G or T, ^{m4}C = 4-methylcytosine, ^{m5}C = 5-methylcytosine, ^{hm5}C = 5-hydroxymethylcytosine, and ^{m6}A = 6-methyladenine

[b] An enzyme is classified as insensitive to methylation if it methylates the methylated sequence at a rate that is at least one tenth the rate at which it methylates the unmethylated sequence An enzyme is classified as sensitive to methylation if it is inhibited at least 20-fold by methylation relative to the unmethylated sequence

[c] *E coli dam* modifies GAT^{m5}C at a reduced rate

Influence of Methylation on DNA Mapping

Mammalian DNA contains ^{m5}C residues in addition to the four normal bases. These residues are found primarily at the 5′ side of G residues. Although only a portion of CpG doublets are methylated, the pattern of methylation is highly cell-type specific (Bird and Southern 1978), any given CpG doublet is methylated in the majority of cells of a given population or in only a few of them.

Nearly all restriction enzymes used for long-range mapping of mammalian chromosomal DNA recognize sequences that contain CpG. Because this dinucleotide occurs approximately fivefold less frequently in mammalian DNA than expected (Normore 1976, Setlow 1976; Shapiro 1976), restriction enzyme recognition sites that contain the CpG dinucleotide are extremely rare (Lindsay and Bird 1987; McClelland and Nelson 1987). Furthermore, most of these dinucleotides are methylated, and almost all enzymes with CpG in the recognition sequence fail to cleave ^{m5}CpG-methylated DNA (Nelson and McClelland 1987); for example, *Bsp*MII, *Cla*I, *Csp*I, *Eag*I, *Mlu*I, *Nae*I, *Nar*I, *Not*I, *Nru*I, *Pvu*I, *Rsr*II, *Sal*I, *Xho*I, and *Xor*II are all sensitive to ^{m5}CpG methylation. Finally, methylation of CpG dinucleotides in preparations of mammalian DNA is rarely complete. This variability in methylation of sites that are recognized by rarely cutting restriction enzymes can be a serious problem in mapping of mammalian DNAs by pulsed-field gel electrophoresis. Among the known restriction enzymes suitable for generating very large fragments of mammalian DNA, only *Acc*III, *Asu*II, *Cfr*9I, *Sfi*I, and *Xma*I can cleave DNA modified at ^{m5}CpG doublets. Propagation of mammalian DNAs in *E. coli* will free CpG dinucleotides from methylation. The pattern of cleavage of the same segment of mammalian genomic DNA will therefore differ in cloned and uncloned preparations.

The CpG dinucleotide is not as rare in many other species as it is in mammalian DNA, and it is not methylated in *Drosophila* and *Caenorhabditis* DNA. Thus, the fragments produced by digestion of these DNAs with rarely cutting restriction enzymes are less than half the size of those produced from mammalian DNA (see Table 5.6).

Enzymes that are *not* sensitive to certain site-specific methylations are particularly useful for achieving complete digestion of modified DNA. For procedures such as the physical mapping of heavily methylated plant DNA, it is desirable to choose restriction enzymes that are *insensitive* to ^{m5}CG and ^{m5}CNG. Examples of such enzymes are *Bcl*I, *Bst*EII, *Bst*NI, *Cvi*QI, *Eco*RV, *Hinc*II, *Hpa*I, *Kpn*I, *Mbo*II, *Nde*I, *Nde*II, *Rsa*I, *Spe*I, *Sph*I, *Taq*I, *Tth*HBI, and *Xmn*I. *Ase*I, *Dra*I, *Mse*I, and *Ssp*I have recognition sequences that do not contain cytosine, so they can be used to cleave heavily cytosine-methylated DNA.

TABLE 5.6 Average Fragment Sizes of Mammalian DNA Produced by Cleavage with Rare Cutting Restriction Enzymes

Enzyme	Recognition sequence	Approximate average fragment size (bp)	Enzyme	Recognition sequence	Approximate average fragment size (bp)
*Not*I	GCGGCCGC	1,000,000–1,500,000	*Eag*I	CGGCCG	100,000
			*Sma*I	CCCGGG	100,000
*Mlu*I	ACGCGT	500,000	*Sal*I	GTCGAC	100,000
*Nru*I	TCGCGA	500,000	*Aat*II	GACGTC	100,000
*Pvu*I	CGATCG	300,000	*Xho*I	CTCGAG	100,000
*Rsr*II	CGGWCCG	200,000	*Sna*BI	TACGTA	100,000
*Sfi*I	GGCCN_5GGCC	200,000	*Fsp*I	TGCGCA	100,000
*Sac*II	CCGCGG	100,000	*Cla*I	ATCGAT	100,000
*Bss*HII	GCGCGC	100,000	*Asu*II	TTCGAA	100,000

These average fragment sizes are the best experimentally determined estimates available and are taken from the data of Brown and Bird (1986), Lindsay and Bird (1987), McClelland and Nelson (1987), and M McClelland (pers comm)

DIGESTING DNA WITH RESTRICTION ENZYMES

Different manufacturers of restriction enzymes recommend significantly different digestion conditions, even for the same restriction enzyme. Because most manufacturers have optimized the reaction conditions for their particular preparations, we recommend following the instructions on the information sheets supplied with the enzymes. Some manufacturers also supply concentrated buffers that have been tested for efficacy with each batch of purified enzyme. These buffers should be used whenever possible

Buffers for different restriction enzymes differ chiefly in the concentration of NaCl that they contain When DNA is to be cleaved with two or more restriction enzymes, the digestions can be carried out simultaneously if both enzymes work well in the same buffer If the enzymes have different requirements, two alternatives are possible· (1) The DNA should be digested first with the enzyme that works best in the buffer of lower ionic strength The appropriate amount of NaCl and the second enzyme can then be added and the incubation continued. (2) A single buffer (potassium glutamate buffer [KGB]) can be used in which virtually all restriction enzymes work (Hanish and McClelland 1988; McClelland et al 1988) The dilutions of this buffer that are used to obtain maximal enzyme activity of various restriction enzymes are given in Table 5.7

TABLE 5.7 Dilutions of KGB and Levels of Enzyme Activity Relative to Those in Manufacturer's Buffer

Enzyme	0.5×	1×	1.5×	2×
*Aat*II	+++	+++	+++	++
*Acc*I	+++	+++	+++	++
*Aha*II	–	++	+	+
*Alu*I	++	+++	++	+
*Apa*LI	+++	+	–	–
*Ava*I	++	++	++	+
*Ava*II	+	+++	++	–
*Avr*II	+++	+++	+++	++
*Bal*I	++	++	+++	+++
*Bam*HI	+++	+++	++	++
*Ban*I	+++	+++	+++	++
*Ban*II	++	+++	+++	++
*Bbv*I	+++	+++	+++	++
*Bcl*I	++	+++	+	–
*Bgl*I	++++	++++	++++	+++
*Bgl*II	++	+++	++	+
*Bsp*1286I	+	+	+	+
*Bsp*MII	+	++	+++	+++
*Bss*HII	+++	+++	++	+
*Bst*EII	+++	+++	+++	+++
*Bst*NI	++	++	++	+
*Bst*XI	+++	+++	++	+
*Cla*I	++	+++	++	++
*Dde*I	++	+++	+++	+++
*Dpn*I	++	++	++	++
*Dra*I	+++	+++	+	+
*Eae*I	+++	+++	++	+
*Eag*I	–	+	++	+++
*Eco*O109I	++	+++	+	+
*Eco*RI	+++	+++	+++	+++
*Eco*RV	++	+++	+++	+++
*Fnu*DII	++	++	+	+
*Fnu*4HI	++	++	++	+
*Fok*I	+++	+++	+	–
*Fsp*I	+++	+++	+++	+
*Hae*II	+++	+++	++	+
*Hae*III	+++	+++	++	++
*Hga*I	+++	+++	++	+
*Hgi*AI	–	+	++	+++
*Hha*I	++	+++	++	+
*Hin*cII	++++	++++	++++	+++
*Hin*dIII	+++	+++	+++	+++
*Hin*fI	++	+++	++	+
*Hpa*I	++	+++	++	+
*Hpa*II	+++	+	+	+
*Hph*I	++	+++	+	–
*Kpn*I	+++	++	–	–
*Mbo*I	++	+++	+++	++++
*Mbo*II	++	+++	+++	++
*Mlu*I	+++	+++	++++	++++
*Msp*I	++	+	+	–
*Mst*II	+++	+++	++	+
*Nar*I	++	+	–	–
*Nci*I	+++	+++	++	+

(continued)

TABLE 5.7 *(continued)*

Enzyme	0.5×	1×	1.5×	2×
*Nco*I	++	+++	+++	+++
*Nde*I	++	+++	++	+
*Nhe*I	+++	+++	+++	++
*Nla*IV	++++	++++	+++	++
*Not*I	++	++	++	++
*Nru*I	++	+++	+++	+++
*Nsi*I	+	+	++	+++
*Pfl*MI	++	+++	++++	+++
*Pst*I	+++	+++	+++	+++
*Pvu*I	++	+++	+++	+++
*Pvu*II	+++	+++	++	+
*Rsa*I	+++	+++	++	+
*Rsr*II	+++	+	–	–
*Sac*I	+++	+++	+	–
*Sac*II	+++	++	++	+
*Sal*I	–	+	+++	+++
*Sau*3AI	++	+++	+++	++++
*Sau*96I	+++	+++	++	+
*Sca*I	+	+++	+++	++
*Scr*FI	+	++	++	++
*Sfa*NI	–	+	++	+++
*Sfi*I	+++	+++	++	+
*Sma*I	+++	+++	+++	++
*Sna*BI	+++	++	+	–
*Ssp*I	++	++	+++	++
*Stu*I	+	++	+++	+++
*Sty*I	++	++	+	+
*Taq*I	++	+++	+++	+
*Xba*I	+++	+++	+++	+
*Xho*I	++	+++	+++	+
*Xmn*I	+++	+++	–	–
Bacteriophage T4 DNA polymerase	++	++	+++	+++
E. coli DNA polymerase I	++	++	+++	+++
Klenow fragment of *E. coli* DNA polymerase I	++	+++	+++	+++
Bacteriophage T4 DNA ligase (1 mM ATP)	+++	++	+	+
Reverse transcriptase	++	++	++	++

(++++) Activity greater than the activity observed in buffer recommended by the manufacturer, (+++) activity approximately equal to the activity observed in buffer recommended by the manufacturer, (++) activity approximately 50–80% of that observed in buffer recommended by the manufacturer, (+) activity approximately 20–50% of that observed in buffer recommended by the manufacturer, (–) activity less than 10% of that observed in buffer recommended by the manufacturer. Bacteriophage T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I both work well in 1.5× and 2× KGB. Bacteriophage T4 DNA ligase works well in 0.5× KGB. (Modified from McClelland et al. 1988.)

Setting Up Digestions with Restriction Enzymes

The following procedure is for a typical reaction containing 0.2–1 μg of DNA. For digestion of larger amounts of DNA, the reaction should be scaled appropriately.

1. Place the DNA solution in a sterile microfuge tube and mix with sufficient water to give a volume of 18 μl

2. Add 2 μl of the appropriate 10 × restriction enzyme digestion buffer (2 × KGB may also be used if the solution volumes are adjusted appropriately.) Mix by tapping the tube.

   2 × KGB

   200 mM potassium glutamate
   50 mM Tris-acetate (pH 7.5)
   20 mM magnesium acetate
   100 μg/ml bovine serum albumin (Fraction V; Sigma)
   1 mM β-mercaptoethanol

3. Add 1–2 units of restriction enzyme, and mix by tapping the tube.

   One unit of enzyme is usually defined as the amount required to digest 1 μg of DNA to completion in 1 hour in the recommended buffer and at the recommended temperature in a 20-μl reaction In general, digestion for longer periods of time or with excess enzyme does not cause problems unless there is contamination with DNAase or exonuclease Such contamination is rare in commercial preparations of restriction enzymes

4. Incubate the mixture at the appropriate temperature for the required period of time

5. Stop the reaction by adding 0 5 M EDTA (pH 8 0) to a final concentration of 10 mM

6. *If the DNA is to be analyzed directly on a gel,* add 6 μl of gel-loading buffer (see Appendix B), mix by vortexing briefly, and load the digest into the gel slot

   If the volume of the restriction enzyme reaction is too large to fit into the slot of a gel, the DNA may be concentrated by the following simple procedure After the reaction has been stopped by the addition of EDTA, add 0 6 volume of 5 M ammonium acetate and 2 volumes of ethanol Store the tube on ice for 5 minutes, and then centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge Discard the supernatant, which contains most of the protein Allow the pellet to dry at room temperature Dissolve the DNA in the appropriate volume of TE (pH 7 6)

   If the digested DNA is to be purified, extract once with phenol chloroform and once with chloroform, and precipitate the DNA with ethanol (see Appendix E).

Notes

Restriction enzymes are expensive! Costs can be kept to a minimum by following the advice given below.

- Many restriction enzymes are supplied by the manufacturer in concentrated form. Often, 1 μl of many enzyme preparations is sufficient to digest 10 μg of DNA in 1 hour To remove small quantities of enzyme from the container, briefly touch the surface of the fluid with the end of a disposable pipette tip. In this way, it is possible to remove as little as 0 1 μl of the enzyme preparation Alternatively, a small piece of narrow-bore plastic tubing (1 cm in length) can be attached to a 1-μl Hamilton syringe and used to transfer a 0.1-μl volume The plastic tubing is discarded after each sample is pipetted

  Concentrated solutions of restriction enzymes may be diluted immediately before use in 1 × restriction enzyme buffer Never dilute an enzyme in water, since it may denature

- Restriction enzymes are stable when they are stored at −20°C in a buffer containing 50% glycerol. When carrying out restriction enzyme digestions, prepare the reactions to the point where all reagents except the enzyme have been mixed Take the enzyme from the freezer, and *immediately* place it on ice *Use a fresh, sterile pipette every time you dispense enzyme.* Contamination of an enzyme with DNA or another enzyme can be costly and can create time-consuming problems Work as quickly as possible, so that the enzyme is out of the freezer for as short a period of time as possible. Return the enzyme to the freezer *immediately* after use.

- Keep reaction volumes to a minimum by reducing the amount of water in the reaction as much as possible However, make sure that the restriction enzyme contributes less than 0 1 volume of the final reaction mixture, otherwise, the enzyme activity may be inhibited by glycerol

- Often, the amount of enzyme can be reduced if the digestion time is increased This can result in considerable savings when large quantities of DNA are cleaved Small aliquots can be removed during the course of the reaction and analyzed on a minigel to monitor the progress of the digestion

- When digesting many DNA samples with the same enzyme, calculate the total amount of enzyme (plus a small excess to allow for the losses involved in transferring several aliquots) that is needed Remove the calculated amount of enzyme solution from the stock, and mix it with the appropriate volume of 1 × restriction enzyme buffer Dispense aliquots of the enzyme/buffer mixture into the reaction mixtures

Other Enzymes Used in Molecular Cloning

Many enzymes other than restriction endonucleases are used routinely in molecular cloning Their properties, enzymatic activities, and, in some cases, reaction conditions are given on the following pages The preparations supplied by commercial manufacturers are of generally excellent quality. However, maximum activity can only be obtained when precautions are taken to stabilize the enzyme in the reaction mixture and when adequate concentrations of substrate(s) are supplied.

- Never use an enzyme in a reaction containing $< 0\ 1$ mg/ml protein because proteins rapidly denature in dilute solutions Bovine serum albumin (Fraction V; Sigma) is a good source of neutral protein to add to enzyme reactions. It should be used at a final concentration of 0 1 mg/ml
- Enzyme reactions work efficiently only when adequate amounts of substrate are present. Always try to work near the K_m (the concentration of substrate that gives half-maximal activity). When using radioactively labeled substrates, calculate the *concentration* of substrate in the reaction from the specific activity provided by the manufacturer If necessary, adjust the concentration of substrate (radioactive plus nonradioactive) until it approaches the K_m of the enzyme
- Enzymes have specific pH, temperature, and ionic requirements Always try to use the optimal reaction conditions

TABLE 5.8 Comparison of Template-dependent DNA Polymerases

	E. coli DNA polymerase I (holoenzyme)	Klenow fragment of *E. coli* DNA polymerase I	Bacteriophage T4 DNA polymerase	Sequenase$^{TM\ a}$ (bacteriophage T7 DNA polymerase)	*Taq* DNA polymerase (AmpliTaq^{TM})	Reverse transcriptase
Reactions						
DNA polymerase 5′ → 3′	+	+	+	+	+	+
Exonuclease						
double-stranded DNA 5′ → 3′	+	–	–	–	+^{b}	–
single-stranded DNA 3′ → 5′	+	+	+	–	–	–
double-stranded DNA 3′ → 5′	+	+	+	–	–	–
RNAase H activity	+	–	–	–	n.i.^{c}	+
Displacement of strand from double-stranded template	+	+	–	–	n.i.	n.i.
Nick translation	+	–	–	–	n.i.	–
Exchange	+	+	+	–	–	–
Template						
Intact double-stranded DNA	–	–	–	–	–	–
Primed single strands of DNA	+	+	+	+	+	+
Duplex with gaps or single-stranded protruding 5′ termini	+	+	+	+	+	+
Nicked double-stranded DNA	+	+	–	–	n.i.	–
Unprimed single-stranded DNA (hairpin)	+	+	n.i.	n.i.	n.i.	+
Single-stranded RNA	+^{d}	+^{d}	–	–	n.i.	+
Requirements						
Divalent cation	Mg^{++e}	Mg^{++}	Mg^{++}	Mg^{++e}	Mg^{++e}	Mg^{++}
pH optimum	7.4	7.4 (phosphate) 8.4 (Tris)	8.0–9.0 (50% as active at pH 7.5)	7.6–7.8	8.3 at room temp (Tris) (50% as active in phosphate [pH 7])	7.6 (MoMLV) 8.3 (AMV)
Sulfhydryl reagents	+	+	+	+	–	+
Structure						
Molecular weight (kD)	109	76	114	92	65	84 (MoMLV) 170 (AMV)
Number of subunits	1	1	1	2 (80, gene *5* product, 12, thioredoxin)	1	1 (MoMLV) 2 (AMV)

See facing page for table notes

DNA POLYMERASES

Many steps in molecular cloning involve the synthesis of DNA in in vitro reactions catalyzed by DNA polymerases. Most of these enzymes require a template and synthesize a product whose sequence is complementary to that of the template. Most polymerases strongly prefer DNA templates, but they will also copy RNA, albeit at lower efficiencies. The most frequently used DNA-dependent DNA polymerases are *E. coli* DNA polymerase I (holoenzyme), the large fragment of *E. coli* DNA polymerase I (Klenow fragment), the DNA polymerases encoded by bacteriophages T4 and T7, modified bacteriophage T7 DNA polymerases (Sequenase™ and Sequenase version 2.0), and thermostable DNA polymerases (*Taq* DNA polymerase and AmpliTaq™). One polymerase, called reverse transcriptase (RNA-dependent DNA polymerase), prefers to copy RNA; it will also accept DNA templates and can therefore be used to synthesize double-stranded DNA copies of RNA templates. Finally, there is one DNA polymerase that does not copy a template at all but adds nucleotides only to the termini of existing DNA molecules. This DNA polymerase is called terminal transferase (terminal deoxynucleotidyl transferase).

The properties of the template-dependent polymerases are summarized in Table 5.8 and described in greater detail in the following pages. The data in Table 5.8 have been collected from numerous papers published over the last 20 years. The values serve as accurate guidelines for using the enzymes, but optimal conditions will always vary slightly with the enzyme preparation (degree of purity) and the DNA preparation or when carrying out a sequence of enzymatic reactions in one mixture.

Note. For a discussion of the relative advantages of various DNA polymerases in sequencing reactions, see Chapter 13.

Notes (Table 5.8)

Table 5.8 is modified from Kornberg and Kornberg (1974) and Lehman (1981).

[a] Sequenase™ is a derivative of bacteriophage T7 DNA polymerase that has been modified by chemical treatment (Sequenase) or by genetic engineering (Sequenase version 2.0) to suppress the 3'→5' exonuclease activity that is a potent component of wild-type bacteriophage T7 DNA polymerase. Consequently, neither version of Sequenase can be used in exchange reactions.

[b] AmpliTaq™ and *Taq* DNA polymerase have a polymerization-dependent 5'→3' exonuclease activity.

[c] n.i. indicates no information available.

[d] Greater activity in the presence of Mn^{++} instead of Mg^{++}.

[e] Mn^{++} may substitute for Mg^{++}, although rates are lower and the specificity of the polymerases may be changed.

DNA Polymerase I (Holoenzyme)
(*E. coli*)

DNA polymerase I consists of a single polypeptide chain ($M_r = 109{,}000$) that can function as a 5′→3′ DNA polymerase, a 5′→3′ exonuclease, and a 3′→5′ exonuclease (Kelley and Stump 1979) and that has an inherent RNAase H activity. The RNAase H activity is essential for cell viability in *E coli* but has not been used in molecular cloning.

USES

1. Labeling of DNA by nick translation (see Figure 5.2) Of all the polymerases, only *E. coli* DNA polymerase I can carry out this reaction, since it alone has a 5′→3′ exonuclease activity that can remove nucleotides from the DNA strand ahead of the advancing enzyme
2. The holoenzyme was originally used for synthesis of the second strand of cDNA in cDNA cloning (Efstratiadis et al 1976), but it has since been superseded by reverse transcriptase and/or the Klenow fragment of *E coli* DNA polymerase I, which do not have 5′→3′ exonuclease activities The 5′→3′ exonuclease of *E coli* DNA polymerase I degrades oligonucleotides that may serve as primers for the synthesis of the second strand of cDNA
3. End-labeling of DNA molecules with protruding 3′ tails This reaction works in two stages First, the 3′→5′ exonuclease activity removes protruding 3′ tails from the DNA and creates a recessed 3′ terminus Then, in the presence of high concentrations of one radiolabeled precursor, exonucleolytic degradation is balanced by incorporation of dNTPs at the 3′ terminus. This reaction, which consists of cycles of removal and replacement of the 3′-terminal nucleotides from recessed or blunt-ended DNA, is sometimes called an exchange or replacement reaction If you plan to use this type of reaction for end-labeling, bacteriophage T4 DNA polymerase is the enzyme of choice Although both *E coli* DNA polymerase I and bacteriophage T4 DNA polymerase can carry out this type of reaction, the bacteriophage enzyme carries a more potent 3′→5′ exonuclease activity.

   In many cases, a single buffer can be used both for cleavage of DNA with a restriction enzyme and for the subsequent end-labeling However, not all restriction enzymes work in buffers used for DNA polymerase reactions, and it is advisable to carry out pilot reactions with the particular batch of enzyme on hand If the restriction enzyme will not work in the DNA polymerase buffer, it is necessary to carry out the restriction enzyme digestion and end-labeling in two separate steps In this case, cleave the DNA in the appropriate restriction enzyme buffer, remove the restriction enzyme by extraction with phenol:chloroform, precipitate the DNA with ethanol, redissolve it in TE, and add the appropriate volume of a 10× DNA polymerase buffer

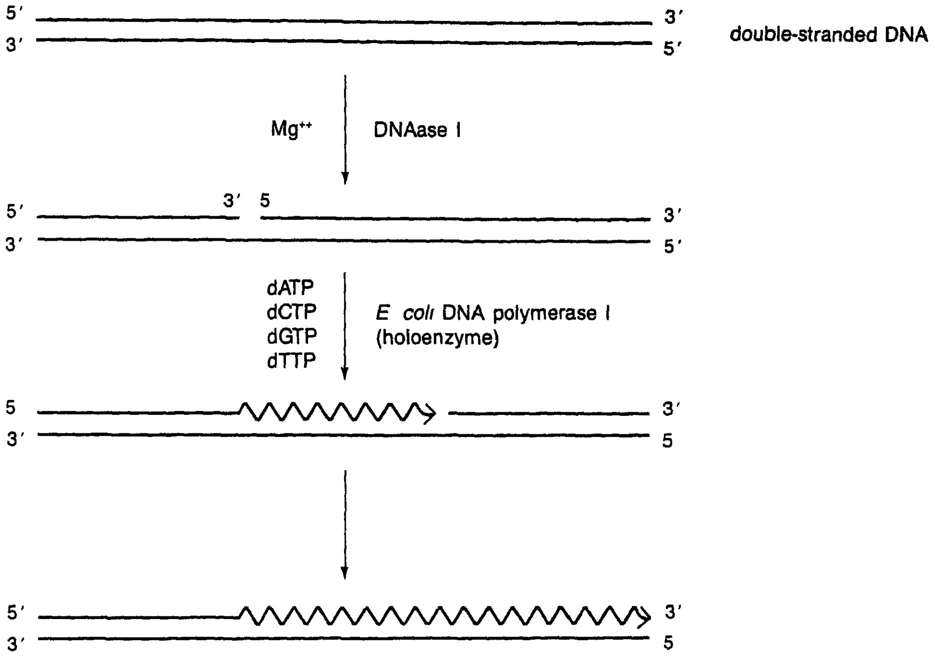


FIGURE 5.2

Nick translation by *E coli* DNA polymerase I Double-stranded DNA is treated with limiting amounts of DNAase I in the presence of magnesium ions The resulting nicks serve as primers for DNA synthesis catalyzed by *E coli* DNA polymerase I (holoenzyme) During synthesis, dNTP precursors are incorporated into the growing chain of DNA and the nick is translated along the DNA in a 5′→3′ direction by virtue of the 5′→3′ exonuclease activity carried by the holoenzyme If radioactive precursors are supplied to the enzyme, the resulting product is radiolabeled and can be used as a hybridization probe (see Chapter 10)

E. coli DNA Polymerase I (Holoenzyme)

Activity: 5′→3′ DNA polymerase

Substrate: Single-stranded DNA template with a DNA primer bearing a 3′-hydroxyl group

Reaction:

$$\mathrm{DNA_{OH}} \xrightarrow[\substack{\mathrm{Mg^{++}} \\ \text{dATP, dTTP, dGTP, dCTP}}]{\substack{\textit{E. coli} \\ \text{DNA polymerase I}}} \mathrm{DNA{-}({}_{p}dN)_{n} + nPP_{i}}$$

For example:

$$\begin{array}{lcr} 5' & {}_{p}C\,{}_{p}C\,{}_{p}G_{\,OH}\ 3' & \\ 3' & G_p\,G_p\,C_p\,T_p\,A_p\,T_p\,C_p\,G_p\,A_p & 5' \end{array}$$

$$\left.\begin{matrix}\mathrm{Mg^{++}} \\ \text{dATP, dTTP,} \\ \text{dGTP, dCTP}\end{matrix}\ \right\downarrow\ \begin{matrix}\textit{E coli} \\ \text{DNA polymerase I}\end{matrix}$$

$$\begin{array}{lcr} 5' & {}_{p}C\,{}_{p}C\,{}_{p}G\,{}_{p}A\,{}_{p}T\,{}_{p}A\,{}_{p}G\,{}_{p}C\,{}_{p}T & 3' \\ 3' & G_p\,G_p\,C_p\,T_p\,A_p\,T_p\,C_p\,G_p\,A_p & 5' \end{array}$$

Activity: 5′→3′ Exonuclease

Substrate: Double-stranded DNA or RNA DNA hybrids. Degrades double-stranded DNA from the 5′ termini, also degrades the RNA component of an RNA DNA hybrid (i.e., this nuclease possesses inherent RNAase H activity)

Reaction:

$$\text{double-stranded DNA} \xrightarrow[\mathrm{Mg^{++}}]{\substack{\textit{E coli} \\ \text{DNA polymerase I}}} \begin{matrix} 5'\,{}_{p}N_{OH} + 5'\,{}_{p}N({}_{p}N)_{n\,p}N_{OH} \\ +\ \text{single-stranded DNA} \end{matrix}$$

For example:

$$\begin{array}{lcr} 5' & {}_{p}C\,{}_{p}G\,{}_{p}C\,{}_{p}A\,{}_{p}T\,{}_{p}C\,{}_{p}T & 3' \\ 3' & G_p\,C_p\,G_p\,C_p\,G_p\,T_p\,A_p\,G_p\,A_p & 5' \end{array}$$

$$\mathrm{Mg^{++}}\ \Big\downarrow\ \begin{matrix}\textit{E coli} \\ \text{DNA polymerase I}\end{matrix}$$

$$\begin{array}{lcr} 5' & {}_{p}C\,{}_{p}A\,{}_{p}T\,{}_{p}C\,{}_{p}T & 3' \\ 3' & G_p\,C_p\,G_p\,C_p\,G_p\,T_p\,A_p\,G_p\,A_p & 5' \end{array}$$

$$+\ 5'\,{}_{p}C + 5'\,{}_{p}G + 5'\,{}_{p}C\,{}_{p}G$$

E. coli DNA Polymerase I (Holoenzyme)

Activity: 3′→5′ Exonuclease

Substrate: Double-stranded or single-stranded DNA containing 3′-hydroxyl termini Degrades DNA from 3′-hydroxyl termini Exonuclease activity on double-stranded DNA is blocked by 5′→3′ DNA polymerase activity and is inhibited by dNMPs with 5′ phosphates

Reaction:

$$\text{double- or single-stranded DNA} \xrightarrow[\text{Mg}^{++}]{\substack{E\ coli \\ \text{DNA polymerase I}}} {}^{5'}{}_{p}N_{OH}$$

For example:

$$\begin{array}{l} 5' \quad {}_pC\,{}_pG\,{}_pC\,{}_pA\,{}_pT\,{}_pC\,{}_pT\;3' \\ 3' \quad G_p\,C_p\,G_p\;5' \end{array}$$

$$\Big\downarrow \text{Mg}^{++} \quad \begin{array}{l} E\ coli \\ \text{DNA polymerase I} \end{array}$$

$$\begin{array}{l} 5' \quad {}_pC\,{}_pG\,{}_pC\,{}_{OH}\;3' \\ 3' \quad G_p\,C_p\,G_p\;5' \\ + {}^{5'}{}_pA + {}^{5'}{}_pC + {}^{5'}{}_pT \end{array}$$

Activity: Exchange (replacement) reaction

Substrate: If only one dNTP is present, the 3′→5′ exonuclease activity will degrade double-stranded DNA from the 3′-hydroxyl terminus until a base is exposed that is complementary to the dNTP A continuous series of synthesis and exchange reactions will then occur at that position

Reaction.

$$\left.\begin{array}{l} 5' \quad {}_pC\,{}_pG\,{}_pT\,{}_pC\,{}_pG\,{}_pC\,{}_{OH}\;3' \\ 3' \quad G_p\,C_p\,A_p\,G_p\,C_p\,G_p\;5' \\ \begin{array}{r} \text{Mg}^{++} \\ [\alpha\text{-}^{32}\text{P}]\text{dTTP} \end{array} \Big\downarrow \begin{array}{l} E\ coli \\ \text{DNA polymerase I} \end{array} \end{array}\right\} \textbf{Exonuclease Activity}$$

$$\left.\begin{array}{l} 5' \quad {}_pC\,{}_pG\,{}_p\;3' \\ 3' \quad G_p\,C_p\,A_p\,G_p\,C_p\,G_p\;5' \\ \qquad\quad \updownarrow \\ 5' \quad {}_pC\,{}_pG\,{}^{*}_{p}T\,{}_{OH}\;3' \\ 3' \quad G_p\,C_p\,A_p\,G_p\,C_p\,G_p\;5' \end{array}\right\} \textbf{DNA Polymerase Activity}$$

Large Fragment of DNA Polymerase I (Klenow Fragment)

(*E. coli*)

The 5′→3′ exonuclease activity of *E. coli* DNA polymerase I is often troublesome because it degrades the 5′ terminus of primers that are bound to DNA templates and removes 5′ phosphates from the termini of DNA fragments that are to be used as substrates for ligation. The 5′→3′ exonuclease activity can be removed proteolytically from the holoenzyme without affecting either the polymerase activity or the 3′→5′ exonuclease activity (Klenow and Henningsen 1970) The Klenow fragment of *E coli* DNA polymerase I that is available today from commercial sources consists of a single polypeptide chain ($M_r = 76{,}000$) produced by cleavage of intact DNA polymerase I with subtilisin or by cloning (Jacobsen et al. 1974, Joyce and Grindley 1983).

USES

1 Filling the recessed 3′ termini created by digestion of DNA with restriction enzymes In many cases, a single buffer can be used both for cleavage of DNA with a restriction enzyme and for the subsequent filling of recessed 3′ termini (or end-labeling of DNA molecules with protruding 3′ tails [see use 3]) The Klenow fragment of *E coli* DNA polymerase I works well in virtually all buffers used for digestion of DNA with restriction enzymes However, not all restriction enzymes work in buffers used for DNA polymerase reactions, and it is advisable to carry out pilot reactions with the particular batch of enzyme on hand. If the restriction enzyme will not work in the DNA polymerase buffer, it is necessary to carry out the restriction enzyme digestion and filling of recessed 3′ termini in two separate steps. In this case, cleave the DNA in the appropriate restriction enzyme buffer, remove the restriction enzyme by extraction with phenol·chloroform, precipitate the DNA with ethanol, redissolve it in TE, and add the appropriate volume of a 10× DNA polymerase buffer.

2 Labeling the termini of DNA fragments by using [^{32}P]dNTPs to fill recessed 3′ termini (end-labeling).

3 End-labeling of DNA molecules with protruding 3′ tails This reaction works in two stages First, the 3′→5′ exonuclease activity removes protruding 3′ tails from the DNA and creates a recessed 3′ terminus. Then, in the presence of high concentrations of one radiolabeled precursor, exonucleolytic degradation is balanced by incorporation of dNTPs at the 3′ terminus This reaction, which consists of cycles of removal and replacement of the 3′-terminal nucleotides from recessed or blunt-ended DNA, is sometimes called an exchange or replacement reaction If you plan to use this type of reaction for end-labeling, bacteriophage T4 DNA polymerase is the enzyme of choice. Although both the Klenow fragment of *E. coli* DNA polymerase I and bacteriophage T4 DNA polymerase can carry out this type of reaction, the bacteriophage enzyme carries a more potent 3′→5′ exonuclease activity

4 Synthesis of the second strand of cDNA in cDNA cloning.

5 Synthesis of double-stranded DNA from single-stranded templates during in vitro mutagenesis.

The Klenow fragment of *E coli* DNA polymerase I can displace hybridized oligonucleotide primers from the template, leading to low frequencies of mutagenesis This problem can be avoided by using bacteriophage T4 DNA polymerase, which does not cause strand displacement (Nossal 1974)

6 Sequencing of DNA using the Sanger dideoxy-mediated chain-termination method (Sanger et al 1977)

7 At one time, the 3′→5′ exonuclease activity of the Klenow fragment of *E coli* DNA polymerase I was used to digest protruding 3′ termini created by some restriction enzymes Lately, bacteriophage T4 DNA polymerase has become the enzyme of choice for this purpose because of its greater 3′→5′ exonuclease activity

8 The Klenow fragment of *E coli* DNA polymerase I has also been used in a polymerase chain reaction to amplify genomic DNA sequences in vitro that are to be used as probes or for direct cloning of mutant alleles of known genes However, the *Taq* DNA polymerase has now become the enzyme of choice for this purpose because it is stable in heat and therefore need not be freshly added after each round of synthesis and denaturation (see page 5.50 and Chapter 14)

Notes

i End-labeling with the Klenow fragment of *E coli* DNA polymerase I provides an alternative to the use of bacteriophage T4 polynucleotide kinase for generating labeled DNA fragments that can be used as size markers during gel electrophoresis Because DNA fragments are labeled in proportion to their molar concentrations and not their sizes, both small and large fragments in a restriction digest become labeled to an equal extent It is therefore possible to use autoradiography to locate bands of DNA that are too small to be visualized by staining with ethidium bromide

ii The end-filling and end-labeling reactions work well on relatively crude DNA preparations (e.g , minipreparations of plasmids)

E. coli DNA Polymerase I Klenow Fragment

Activity: 5′→3′ DNA polymerase

Substrate: Single-stranded DNA template with a primer containing a free 3′-hydroxyl group

Reaction:

$$DNA_{OH} \xrightarrow[\substack{Mg^{++}\\ dATP,\ dTTP,\ dGTP,\ dCTP}]{\substack{\text{Klenow fragment}\\ \text{of } E\ coli\\ \text{DNA polymerase I}}} DNA\text{-}({}_{p}dN)_{n} + nPP_{i}$$

For example:

$$\begin{array}{lll}
5' & {}_{p}C\ {}_{p}C\ {}_{p}G\ {}_{OH}\ 3' & \\
3' & G_{p}\ G_{p}\ C_{p}\ T_{p}\ A_{p}\ T_{p}\ C_{p}\ G_{p}\ A_{p} & 5'
\end{array}$$

$$\begin{array}{rcl}
\begin{array}{r} Mg^{++}\\ dATP,\ dTTP,\\ dGTP,\ dCTP \end{array} & \Big\downarrow & \begin{array}{l} \text{Klenow fragment of}\\ E\ coli \text{ DNA polymerase I} \end{array}
\end{array}$$

$$\begin{array}{lll}
5' & {}_{p}C\ {}_{p}C\ {}_{p}G\ {}_{p}A\ {}_{p}T\ {}_{p}A\ {}_{p}G\ {}_{p}C\ {}_{p}T & 3'\\
3' & G_{p}\ G_{p}\ C_{p}\ T_{p}\ A_{p}\ T_{p}\ C_{p}\ G_{p}\ A_{p} & 5'
\end{array}$$

Activity. 3′→5′ Exonuclease

Substrate Double- or single-stranded DNA degrades from free 3′-hydroxyl termini, exonuclease activity on double-stranded DNAs is blocked by 5′→3′ polymerase activity

Reaction.

$$\text{double- or single-stranded DNA} \xrightarrow[Mg^{++}]{\substack{\text{Klenow fragment}\\ \text{of } E\ coli\\ \text{DNA polymerase I}}} {}^{5'}{}_{p}N_{OH}$$

For example

$$\begin{array}{ll}
5' & {}_{p}C\ {}_{p}G\ {}_{p}C\ {}_{p}A\ {}_{p}T\ {}_{p}C\ {}_{p}T\ 3'\\
3' & G_{p}\ C_{p}\ G_{p}\ 5'
\end{array}$$

$$\begin{array}{rcl}
Mg^{++} & \Big\downarrow & \begin{array}{l} \text{Klenow fragment of}\\ E\ coli \text{ DNA polymerase I} \end{array}
\end{array}$$

$$\begin{array}{ll}
5' & {}_{p}C\ {}_{p}G\ {}_{p}C\ {}_{OH}\ 3'\\
3' & G_{p}\ C_{p}\ G_{p}\ 5'
\end{array}$$

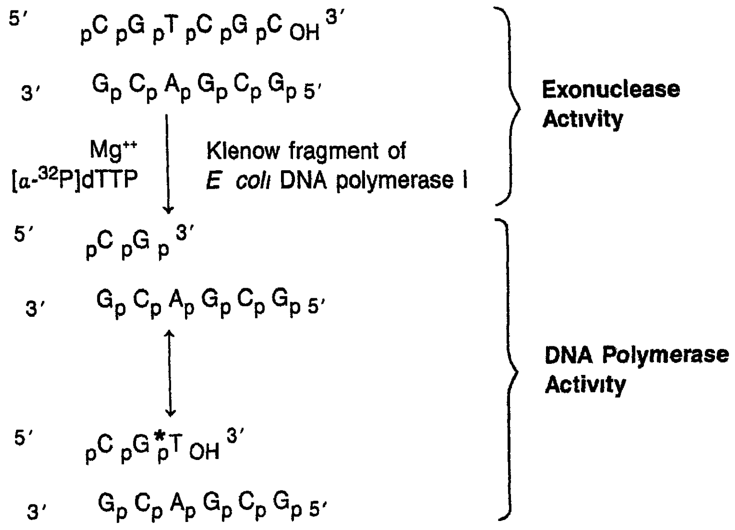
$$+\ {}^{5'}{}_{p}A + {}^{5'}{}_{p}C + {}^{5'}{}_{p}T$$

E. coli DNA Polymerase I Klenow Fragment

Activity: Exchange (replacement) reaction

Substrate: If only one dNTP is present, the 3′→5′ exonuclease activity will degrade double-stranded DNA from the 3′-hydroxyl terminus until a base is exposed that is complementary to the dNTP A continuous series of synthesis and exchange reactions will then occur at that position

Reaction:



Bacteriophage T4 DNA Polymerase

(Bacteriophage T4-infected *E. coli*)

Bacteriophage T4 DNA polymerase ($M_r = 114{,}000$) and the Klenow fragment of *E. coli* DNA polymerase I are similar in that each possesses a 5′→3′ polymerase activity and a 3′→5′ exonuclease activity that is more active on single-stranded DNA than on double-stranded DNA However, the exonuclease activity of bacteriophage T4 DNA polymerase is more than 200 times that of the Klenow fragment Since it does not displace oligonucleotide primers from single-stranded DNA templates, bacteriophage T4 DNA polymerase works more efficiently than the Klenow fragment of *E. coli* DNA polymerase I in mutagenesis reactions in vitro (Nossal 1974).

USES

1 Filling or labeling the recessed 3′ termini created by digestion of DNA with restriction enzymes Labeling reactions must be carried out in the presence of high concentrations of dNTPs in order for the polymerization (filling) reaction to overwhelm the powerful 3′→5′ exonuclease activity

2. End-labeling of DNA molecules with protruding 3′ tails This reaction works in two stages First, the potent 3′→5′ exonuclease activity removes protruding 3′ tails from the DNA and creates a recessed 3′ terminus Then, in the presence of high concentrations of one radiolabeled precursor, exonucleolytic degradation is balanced by incorporation of dNTPs at the 3′ terminus. This reaction, which consists of cycles of removal and replacement of the 3′-terminal nucleotides from recessed or blunt-ended DNA, is sometimes called an exchange or replacement reaction.

   In many cases, a single buffer can be used both for cleavage of DNA with a restriction enzyme and for the subsequent end-labeling. Bacteriophage T4 DNA polymerase will function at about 50% of maximal activity in all buffers that are commonly used for digestion of DNA with restriction enzymes However, not all restriction enzymes work in buffers used for DNA polymerase reactions, and it is advisable to carry out pilot reactions with the particular batch of enzyme on hand If the restriction enzyme will not work in the DNA polymerase buffer, it is necessary to carry out the restriction enzyme digestion and end-labeling in two separate steps. In this case, cleave the DNA in the appropriate restriction enzyme buffer, remove the restriction enzyme by extraction with phenol:chloroform, precipitate the DNA with ethanol, redissolve it in TE, and add the appropriate volume of a 10× DNA polymerase buffer. Filling and end-labeling reactions with bacteriophage T4 DNA polymerase can be carried out at 12°C to maximize the ratio of polymerase activity to exonuclease activity. However, these reactions are often carried out at room temperature or at 37°C without adverse effects

3. Labeling DNA fragments for use as hybridization probes. The recessed 3′ termini created by partial digestion of double-stranded DNA with the 3′→5′ exonuclease activity are filled with [^{32}P]dNTPs (replacement synthesis) (O'Farrell et al. 1980). Hybridization probes prepared by this technique have two advantages over probes prepared by nick translation. First, they lack the artifactual hairpin structures that can be produced

during nick translation. Second, they can easily be converted into strand-specific probes by cleavage with suitable restriction enzymes (see Figure 5.3).

However, in contrast to nick translation, this method does not produce a uniform distribution of label along the length of the DNA. Furthermore, the 3′ exonuclease activity degrades single-stranded DNA much faster than it degrades double-stranded DNA, so that after a molecule has been digested to its midpoint, it will dissociate into two half-length single strands that will be rapidly degraded It is therefore important to stop the exonuclease reaction before the enzyme reaches the center of the molecule. Consequently, the replacement synthesis method yields a population of molecules that are fully labeled at their termini but contain progressively decreasing quantities of label toward their centers.

4 Conversion of termini of double-stranded DNA to blunt-ended molecules Protruding 3′ termini will be removed from double-stranded DNA by the potent 3′→5′ exonuclease activity of bacteriophage T4 DNA polymerase In the presence of high concentrations of dNTPs, further degradation of the double-stranded region of the template will be balanced by synthesis The ability to convert protruding 3′ termini to blunt ends is an extremely

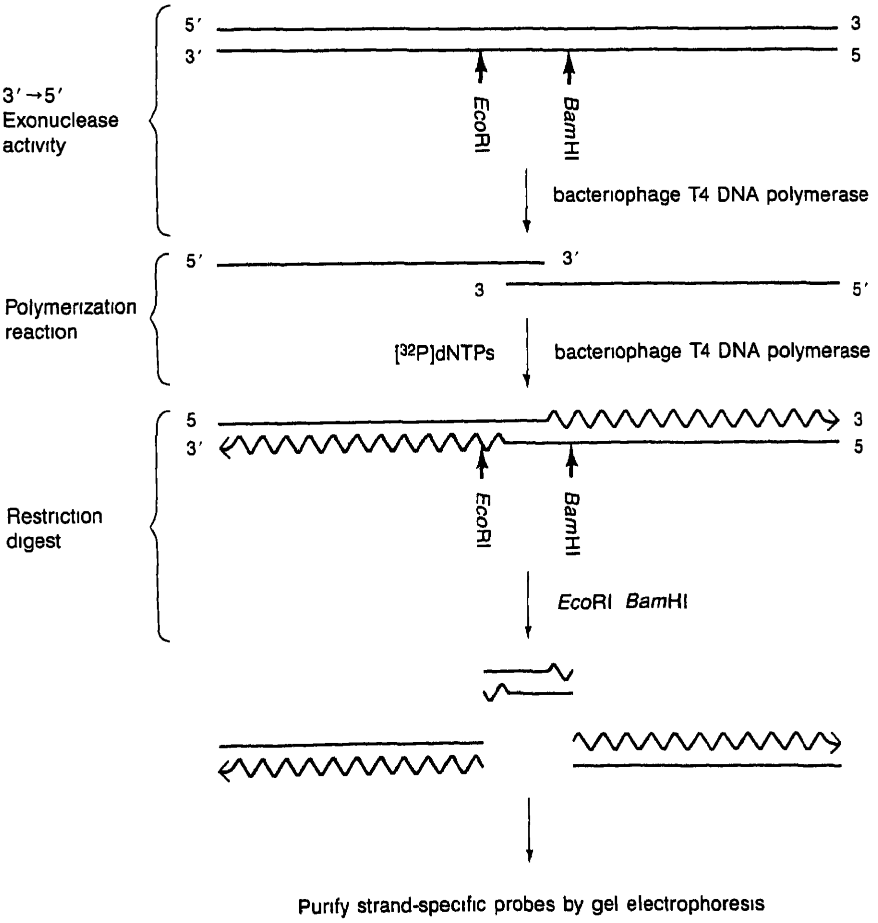

3′→5′ Exonuclease activity
5′
3′
3
5
EcoRI
BamHI
bacteriophage T4 DNA polymerase
Polymerization reaction
5′
3′
3
5′
[32P]dNTPs
bacteriophage T4 DNA polymerase
Restriction digest
5
3′
3
5
EcoRI
BamHI
EcoRI BamHI
Purify strand-specific probes by gel electrophoresis

FIGURE 5.3
Production of strand-specific hybridization probes

Bacteriophage T4 DNA Polymerase

Activity: 5′→3′ DNA polymerase

Substrate: 3′-hydroxyl, single-stranded DNA template-primer complex The enzyme cannot displace the strand ahead of the nick. However, addition of bacteriophage T4 gene *32* protein allows the enzyme to begin synthesis at a nick in buffers of low ionic strength

Reaction:

$$\mathrm{DNA_{OH}} \xrightarrow[\substack{\mathrm{Mg^{++}} \\ \text{dATP, dTTP, dGTP, dCTP}}]{\substack{\text{bacteriophage T4} \\ \text{DNA polymerase}}} \mathrm{DNA{-}({}_pdN)_n + nPP_i}$$

For example:

$$\begin{array}{lll} 5' & {}_pC\ {}_pC\ {}_pG\ {}_{OH}\ 3' & \\ 3' & G_p\ G_p\ C_p\ T_p\ A_p\ T_p\ C_p\ G_p\ A_p & 5' \end{array}$$

$$\begin{array}{r} \mathrm{Mg^{++}} \\ \text{dATP, dTTP,} \\ \text{dGTP, dCTP} \end{array} \Bigg\downarrow \begin{array}{l} \text{bacteriophage T4} \\ \text{DNA polymerase} \end{array}$$

$$\begin{array}{lll} 5' & {}_pC\ {}_pC\ {}_pG\ {}_pA\ {}_pT\ {}_pA\ {}_pG\ {}_pC\ {}_pT & 3' \\ 3' & G_p\ G_p\ C_p\ T_p\ A_p\ T_p\ C_p\ G_p\ A_p & 5' \end{array}$$

Activity. 3′→5′ Exonuclease

Substrate: Considerably more active on single-stranded DNA than on double-stranded DNA, exonuclease activity on double-stranded DNA is blocked by 5′→3′ DNA polymerase activity

Reaction:

$$\text{single-stranded DNA} \xrightarrow[\mathrm{Mg^{++}}]{\substack{\text{bacteriophage T4} \\ \text{DNA polymerase}}} {}^{5'}{}_pN_{OH}$$

For example:

$$\begin{array}{ll} 5' & {}_pC\ {}_pG\ {}_pC\ {}_pA\ {}_pT\ {}_pC\ {}_pT\ 3' \\ 3' & G_p\ C_p\ G_p\ 5' \end{array}$$

$$\mathrm{Mg^{++}} \Bigg\downarrow \begin{array}{l} \text{bacteriophage T4} \\ \text{DNA polymerase} \end{array}$$

$$\begin{array}{ll} 5' & {}_pC\ {}_pG\ {}_pC\ {}_{OH}\ 3' \\ 3' & G_p\ C_p\ G_p\ 5' \end{array}$$

$$+\ {}^{5'}{}_pA + {}^{5'}{}_pC + {}^{5'}{}_pT$$

valuable reaction that is frequently used when preparing DNAs for addition of synthetic linkers As described above, molecules with recessed 3′ termini can be repaired by bacteriophage T4 DNA polymerase in a filling reaction similar to that catalyzed by the Klenow fragment of *E coli* DNA polymerase I Thus, DNAs with a mixture of protruding 5′ and 3′ termini (e g , double-stranded cDNAs synthesized from RNA templates) can be converted to blunt-ended molecules (polished) by bacteriophage T4 DNA polymerase in the presence of high concentrations of dNTPs

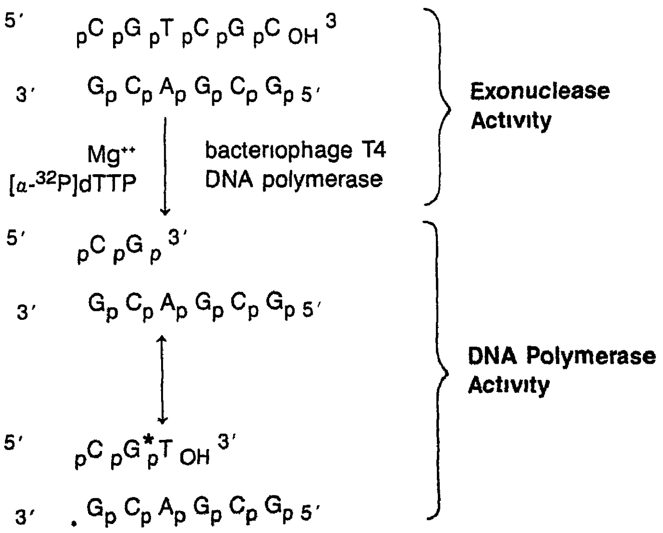
5 Extension of mutagenic oligonucleotide primers that are bound to single-stranded DNA templates Bacteriophage T4 DNA polymerase is preferred in this reaction to the Klenow fragment of *E coli* DNA polymerase I because it cannot displace the short oligonucleotide from the template The efficiency of mutagenesis is therefore increased approximately twofold

Bacteriophage T4 DNA Polymerase

Activity: Exchange (replacement) reaction

Substrate. If only one dNTP is present, the 3′→5′ exonuclease activity will degrade double-stranded DNA from the 3′-hydroxyl terminus until a base is exposed that is complementary to the dNTP A continuous series of synthesis and exchange reactions will then occur at that position

Reaction:



Bacteriophage T7 DNA Polymerase

(Bacteriophage T7-infected *E. coli*)

The DNA polymerase induced after infection of *E coli* by bacteriophage T7 is a complex of two tightly bound proteins, the bacteriophage T7 gene *5* protein and the host protein thioredoxin This complex is the most processive of all known DNA polymerases Thus, the average length of DNA synthesized by a single molecule of bacteriophage T7 DNA polymerase is much greater than that of DNAs synthesized by other DNA polymerases This has considerable advantages in certain circumstances, for example, when sequencing DNA by the Sanger dideoxy chain-termination method (Sanger et al 1977) (see Chapter 13) Bacteriophage T7 DNA polymerase, like the Klenow fragment and the holoenzyme of *E. coli* DNA polymerase I and bacteriophage T4 DNA polymerase, has a 3′→5′ exonuclease activity (encoded by bacteriophage T7 gene *5*). As in bacteriophage T4 DNA polymerase, the 3′→5′ exonuclease activity is potent. The degree of activity of the 3′→5′ exonuclease of bacteriophage T7 DNA polymerase is approximately 1000 times that of the Klenow fragment of *E. coli* DNA polymerase I Bacteriophage T7 DNA polymerase does *not* have a 5′→3′ exonuclease activity (Tabor et al 1987) See pages 5 46–5.47 for illustrations of the 5′→3′ DNA polymerase and 3′→5′ exonuclease activities and exchange reaction encoded by bacteriophage T7 DNA polymerase.

USES

1 Primer-extension reactions that require the copying of long stretches of template

2 Rapid end-labeling by either filling or exchange (replacement) reactions such as those described for bacteriophage T4 DNA polymerase

Modified Bacteriophage T7 DNA Polymerase (Sequenase™)

The bacteriophage T7 gene *5* protein ($M_r = 80{,}000$) consists of two separate domains, one of which bears a high degree of homology with the DNA-binding and polymerization domain of *E. coli* DNA polymerase I (Ollis et al 1985a,b). This binding/polymerization domain appears to occupy most of the carboxy-terminal sequences of the molecule, whereas the amino terminus carries the powerful 3′→5′ exonuclease activity. The exonuclease may be inactivated by incubating the enzyme for several days with a reducing agent, molecular oxygen, and low concentrations of ferrous ions This inactivation is thought to be the result of site-specific modifications mediated by reactive oxygen radicals that are generated by ferrous ions bound to the protein Over 99% of the 3′→5′ exonuclease may be removed without appreciably affecting the polymerizing ability of the protein (Tabor and Richardson 1987b). The resulting gene *5* protein/thioredoxin complex (modified bacteriophage T7 DNA polymerase) is highly processive and is therefore ideal for determining the sequence of long tracts of DNA by the dideoxy-mediated chain-termination method (Sanger et al 1977) (see Chapter 13) Chemically modified bacteriophage T7 DNA polymerase is marketed under the trade name Sequenase by United States Biochemical (Tabor and Richardson 1987a) Recently, an improved version of Sequenase (2 0) that completely lacks exonuclease activity has been produced by genetic engineering See page 5 46 for an illustration of the 5′→3′ DNA polymerase activity encoded by Sequenases

Taq DNA Polymerase and AmpliTaq™

(*Thermus aquaticus*)

Taq DNA polymerase is a thermostable DNA-dependent DNA polymerase ($M_r = 65{,}000$) originally purified from the extreme thermophile *Thermus aquaticus* (Chien et al 1976) and now available in a genetically engineered form (AmpliTaq™; Perkin Elmer Cetus) (Saiki and Gelfand 1989). These enzymes have a $5' \rightarrow 3'$ polymerization-dependent exonuclease activity. For nucleotide incorporation, the enzyme works best at 75–80°C, depending on the target sequence, its polymerase activity is reduced by a factor of 2 at 60°C and by a factor of 10 at 37°C. In many cases, however, it is necessary to initiate polymerization reactions at suboptimal temperatures to prevent dissociation of the primer from the template.

USE

Taq DNA polymerase is used for DNA sequencing (Chapter 13) and to amplify specific sequences of DNA in vitro by the polymerase chain reaction (Chapter 14) This is accomplished using two oligonucleotide primers complementary to sequences that flank the region of interest. The first oligonucleotide is complementary to sequences of one strand of the template upstream of the region of interest, and the second is complementary to the other strand of the template downstream from the region of interest. The denatured template is incubated in the presence of a vast molar excess of both primers to generate primer templates that serve as substrates for the *Taq* DNA polymerase After completion of synthesis, the reaction mixture is heated to denature the newly synthesized DNA and reincubated to allow the primers to reanneal Because the heat-stable *Taq* DNA polymerase is not inactivated during the denaturation step, a second round of specific synthesis can occur without the addition of either new enzyme or new primers. Successive cycles of denaturation, annealing, and polymerization result in exponential amplification of the sequences that lie between the two primers (Saiki et al 1985).

This repetitive procedure, which can use either genomic DNA or single-stranded cDNA as the original template, can result in a 10^6-fold amplification of specific sequences It is therefore extremely useful for mapping and sequencing mutations in uncloned genomic DNA or mRNA (Saiki et al 1985; Kogan et al 1987) and for direct cloning of the desired genomic DNA or mRNA sequences (Scharf et al 1986) Because of the tremendous amplifications that are achieved, the method requires minute amounts of template For example, enough genomic DNA can be obtained from approximately 100 μl of blood to allow unambiguous determination of the presence or absence of a particular mutation at a specific site in a particular segment of DNA

Notes

i *Taq* DNA polymerase requires Mg^{++}. Because the melting and annealing temperatures of the primer:template hybrids are affected by the presence of divalent cations, it is often necessary to determine the optimal concentration of Mg^{++} for each set of amplification reactions

ii Phosphate buffers inhibit *Taq* DNA polymerase and should be avoided. This reaction is usually carried out in a Tris buffer at a room temperature pH of 8.3.

Taq DNA Polymerase

Activity: 5′→3′ DNA polymerase

Substrate: Single-stranded template, primer with 3′ hydroxyl

Reaction:

$$\mathrm{DNA_{OH}} \xrightarrow[\substack{\mathrm{Mg^{++}} \\ \mathrm{dATP,\ dTTP,\ dGTP,\ dCTP}}]{\textit{Taq}\ \mathrm{DNA\ polymerase}} \mathrm{DNA{-}({}_{p}dN)_{n} + nPP_{i}}$$

Reverse Transcriptase (RNA-dependent DNA Polymerase)

(Murine and avian retroviruses)

Two forms of reverse transcriptase are commercially available: a preparation made from purified avian myeloblastosis virus (AMV) and an enzyme isolated from a strain of *E. coli* that expresses a cloned copy of the reverse transcriptase gene of the Moloney murine leukemia virus (Mo-MLV). Both enzymes lack a 3' → 5' exonuclease active on DNA and catalyze the reactions shown on page 5 55.

The murine and avian reverse transcriptases differ from one another in a number of respects·

- The avian enzyme consists of two polypeptide chains that carry both a polymerase activity and a powerful RNAase H activity (Verma 1981) The murine enzyme, a single polypeptide chain of $M_r = 84,000$, has a polymerase activity and a comparatively weak RNAase H activity (Gerard 1983) This weak RNAase activity is a considerable advantage when attempting to synthesize cDNAs complementary to long mRNAs. At the beginning of the reaction, the hybrids formed between the primer and the template mRNA are substrates for RNAase H. Thus, at the beginning of cDNA synthesis, there is a competition between degradation of the template mRNA and initiation of DNA synthesis (Berger et al 1983) In addition, RNAase H can cleave the template near the 3' terminus of the growing DNA strand if reverse transcriptase pauses during synthesis (Kotewicz et al. 1988). Thus, the high level of RNAase H activity in preparations of the avian enzyme tends to suppress the yield of cDNA and to restrict its length
- The avian enzyme works efficiently at 42°C (the normal body temperature of chickens), whereas the murine enzyme is rapidly inactivated at this temperature. RNAs rich in secondary structure are therefore copied more efficiently by the avian enzyme than by the murine enzyme However, preparations of the avian enzyme can be contaminated by an endonuclease that cleaves DNA This is now less of a problem than it was in the early 1980s, when cDNA libraries generated with comparatively impure preparations of the avian enzyme seldom exceeded 1 kb in length
- The avian enzyme works more efficiently at pH 8 3 than at pH 7 6, the pH preferred by the murine enzyme The length of the cDNA synthesized by either enzyme is greatly reduced when reactions are carried out at a pH that differs from the optimum by as little as 0.2 unit Since the pH of Tris changes with temperature, it is essential to check that the pH of the reaction mixture is correct at the temperature chosen for incubation

USES

1. Reverse transcriptase is used chiefly to transcribe mRNA into double-stranded cDNA that can be inserted into prokaryotic vectors. However, reverse transcriptase can also be used with either single-stranded DNA or RNA templates to make probes for use in hybridization experiments. Three types of primers are used in these reactions.
   - *Oligo(dT)*$_{12-18}$, which binds to the poly(A) tract at the 3' terminus of

mammalian mRNAs and primes the synthesis of the first strand of cDNA. Depending on the quality of the reverse transcriptase and the reaction conditions, the sequences at the 3′ terminus of the template may be overrepresented in the cDNA.

- *Oligonucleotides of random sequence* (Taylor et al. 1976). The aim is to use a population of oligonucleotides whose sequence diversity is so large that at least some individual oligonucleotides will anneal to the template and serve as primers for reverse transcriptase Because different oligonucleotides will bind to different sequences, a large proportion of the sequences of the template will be copied by the enzyme, and if all of the primers are present at equal concentrations, all sequences of the template should be copied at equal frequencies.

  Note Oligonucleotides of random sequence can be synthesized on an automated DNA synthesizer or can be generated by hydrolysis of high-molecular-weight DNA (see Chapter 11)

- *Oligonucleotides of defined sequence* Oligonucleotides of defined sequence can be used to prime the synthesis of cDNA corresponding to a particular mRNA Because the newly synthesized DNA is complementary to the sequences of the mRNA that lie upstream of the primer, this method (primer extension) provides an accurate measurement of the distance between a fixed point on an mRNA and its 5′ terminus

2 Labeling the termini of DNA fragments with protruding 5′ termini (filling reaction).

3. The enzyme can also be used to sequence DNAs by the dideoxy chain-termination method (Sanger et al. 1977) when other enzymes (e.g., the Klenow fragment of *E coli* DNA polymerase I or Sequenase) yield unsatisfactory results

Notes

i Reverse transcriptase lacks $3' \rightarrow 5'$ exonuclease activity, which acts as an editing function in *E coli* DNA polymerase I, and is therefore prone to error In the presence of high concentrations of dNTPs and Mn^{++}, about 1 base in every 500 is misincorporated

ii The K_m of reverse transcriptase for its dNTP substrates is very high—in the millimolar range To prevent premature termination of newly synthesized DNA chains, it is therefore essential to include high concentrations of dNTPs in the reaction

iii. Reverse transcriptase can be used to synthesize single-stranded copies of DNA templates using oligonucleotide primers (see Figure 5.4A). However, both double-stranded and single-stranded cDNAs are generated from RNA templates (see Figure 5.4B). Self-primed synthesis is much less efficient than synthesis from the added oligonucleotide primers. Therefore, self-complementary hairpin molecules usually constitute only a small fraction of the synthesized double-stranded cDNA (see Chapter 8). If necessary, both self-primed and exogenously primed second-strand synthesis can be inhibited by including actinomycin D in the reaction mixture at a final concentration of 50 μg/ml.

Caution: Actinomycin D is a teratogen and carcinogen. Stock solutions should be prepared, wearing gloves and a mask, in a chemical hood, not on an open bench. Solutions of actinomycin D are light-sensitive Instructions for preparing and storing stock solutions of actinomycin D are given in Appendix B.

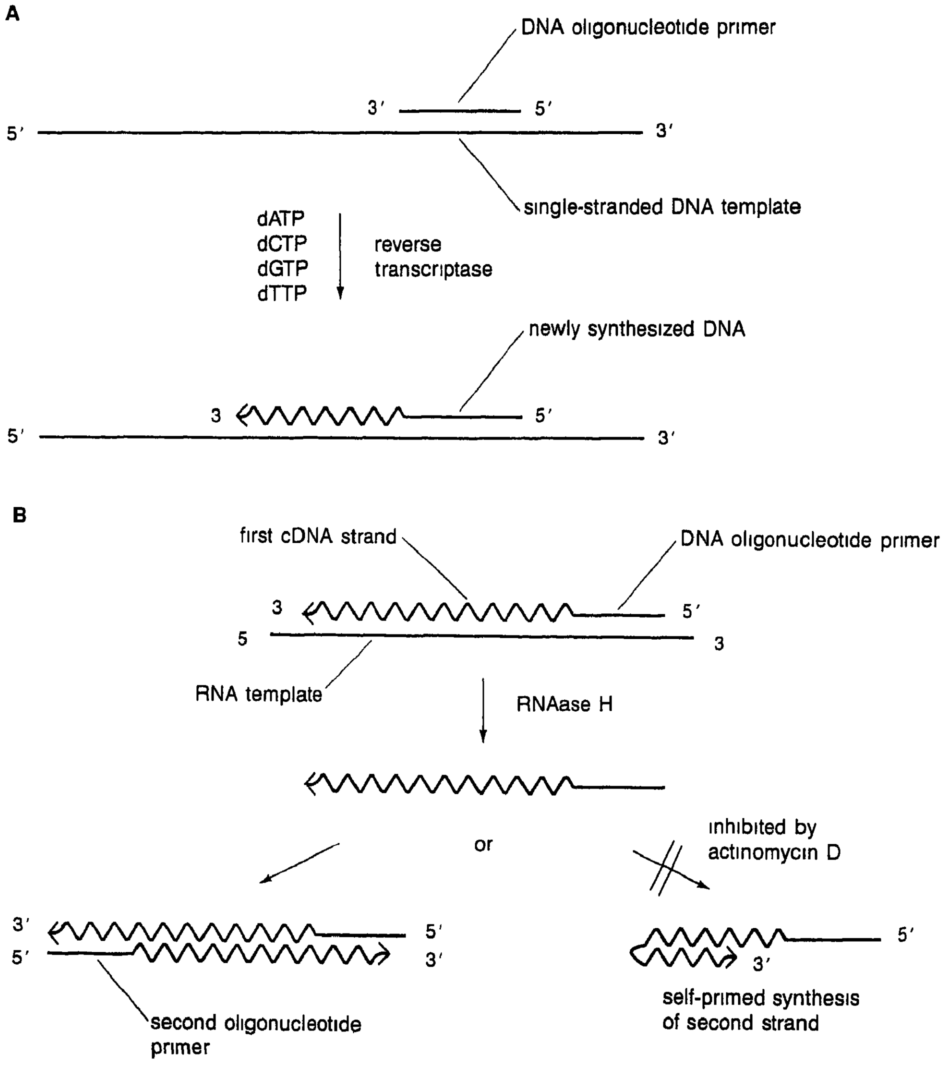


FIGURE 5.4
The use of reverse transcriptase to generate probes from (*A*) DNA and (*B*) RNA templates.

Reverse Transcriptase

Activity: 5′→3′ DNA polymerase

Substrate: RNA or DNA template with an RNA or DNA primer bearing a 3′-hydroxyl group

Reaction:

$$\mathrm{DNA_{OH}\ or\ RNA_{OH}} \xrightarrow[\substack{\mathrm{Mg^{++}} \\ \mathrm{dATP,\ dTTP,\ dGTP,\ dCTP}}]{\text{reverse transcriptase}} \begin{array}{l} \mathrm{DNA{-}({}_pdN)_n + nPP_i} \\ \mathrm{RNA{-}({}_pdN)_n + nPP_i} \end{array}$$

For example:

$$\begin{array}{ll} 5' & \mathrm{{}_pT\ {}_pT\ {}_pT\ {}_pT\ {}_pT_{OH}}\ 3' \\ 3' & \mathrm{A_p\ A_p\ A_p\ A_p\ A_p\ U_p\ C_p\ U_p\ G_p\ U_p\ C_p\ C_p\ U_p\ A_p}\ 5' \end{array}$$

$$\Big\downarrow \begin{array}{l} \text{Mg}^{++} \\ \text{dATP, dTTP,} \\ \text{dGTP, dCTP} \end{array} \quad \text{reverse transcriptase}$$

$$\begin{array}{ll} 5' & \mathrm{{}_pT\ {}_pT\ {}_pT\ {}_pT\ {}_pT\ {}_pA\ {}_pG\ {}_pA\ {}_pC\ {}_pA\ {}_pG\ {}_pG\ {}_pA\ {}_pT_{OH}}\ 3' \\ 3' & \mathrm{A_p\ A_p\ A_p\ A_p\ A_p\ U_p\ C_p\ U_p\ G_p\ U_p\ C_p\ C_p\ U_p\ A_p}\ 5 \end{array}$$

Activity: RNAase H (5′→3′ and 3′→5′ exoribonuclease)

Substrate. Reverse transcriptase specifically degrades RNA in an RNA DNA hybrid by a processive mechanism

Reaction. Degradation of substrates with free ends yielding ribonucleotide products that are 4–20 nucleotides in length and contain 5′-phosphate and 3′-hydroxyl termini

$$\begin{array}{lll} \text{RNA} & 5' & \mathrm{{}_pU\ {}_pC\ {}_pC\ {}_pG\ {}_pU\ {}_pA}\ 3' \\ \text{DNA} & 3' & \mathrm{A_p\ G_p\ G_p\ C_p\ A_p\ T_5} \end{array} \xrightarrow[\text{transcriptase}]{\text{reverse}} \begin{array}{ll} 5 & \mathrm{{}_pU\ {}_pC}\ 3' \\ 3 & \mathrm{A_p\ G_p\ G_p\ C_p\ A_p\ T}\ 5' \\ & +\ 5'\ \mathrm{{}_pC\ {}_pG\ {}_pU\ {}_pA}\ 3' \end{array}$$

Terminal Transferase (Terminal Deoxynucleotidyl Transferase)

(Calf thymus)

Terminal transferase ($M_r = 60{,}000$) is an unusual DNA polymerase found only in prelymphocytes and in early stages of lymphoid differentiation (Chang and Bollum 1986). In the presence of a divalent cation, the enzyme catalyzes the addition of dNTPs to the 3′-hydroxyl termini of DNA molecules (Bollum 1974) When the nucleotide to be added is a purine, Mg^{++} is the preferred cation, when the nucleotide is a pyrimidine, Co^{++} is used instead. The minimum chain length of the acceptor DNA is three dNTPs, and as many as several thousand dNTPs can be incorporated if the ratio of acceptor to nucleotide is adjusted correctly. Single nucleotides can be added to the 3′ termini of DNA if modified nucleotides (e g., ddNTPs or cordycepin triphosphate) are used as substrates Homopolymers of rNTPs can also be synthesized at the 3′ termini of DNA molecules in the presence of Co^{++} (for references, see Chang and Bollum 1986). The enzyme strongly prefers to use DNAs with protruding 3′ termini as acceptors However, blunt or recessed 3′ termini are used, albeit less efficiently, in buffers of low ionic strength that contain Co^{++}, Mg^{++}, or Mn^{++} (Roychoudhury et al 1976; Nelson and Brutlag 1979; Roychoudhury and Wu 1980; Michelson and Orkin 1982, Deng and Wu 1983)

USES

1 Addition of complementary homopolymeric tails to vector and cDNA

2 Labeling the 3′ termini of DNA fragments with a ^{32}P-labeled dNTP (Tu and Cohen 1980), a ddNTP (Cozzarelli et al 1969), or an rNTP (Wu et al. 1976) For labeling with rNTPs, [α-^{32}P]rNTP is used in the presence of Co^{++}, followed by treatment with alkali

Terminal Transferase

Activity: Terminal transferase

Substrate: Single-stranded DNA with a 3′-hydroxyl terminus or double-stranded DNA with a protruding 3′-hydroxyl terminus is preferred. Blunt-ended, double-stranded DNA or DNA with a recessed 3′-hydroxyl terminus serves as a template if Co^{++} is supplied as a cofactor (Roychoudhury et al 1976, Nelson and Brutlag 1979, Roychoudhury and Wu 1980, Michelson and Orkin 1982, Deng and Wu 1983).

Reaction:

$$\begin{tikzcd} \text{single-stranded DNA}_{\text{OH}} \arrow[d, "\text{terminal transferase}", "\begin{array}{l}\text{Mn}^{++}\text{ or Mg}^{++}\\ \text{dATP, dTTP,}\\ \text{dGTP, dCTP}\end{array}"'] \\ \text{DNA}-({}_{p}\text{dN})_{n} + n\text{PP}_{i} \end{tikzcd}$$

DNA-DEPENDENT RNA POLYMERASES

Bacteriophage SP6 and Bacteriophages T7 and T3 RNA Polymerases

(Bacteriophage SP6-infected *Salmonella typhimurium* LT2
and bacteriophage T7- or T3-infected *E. coli*)

Bacteriophage SP6 synthesizes a DNA-dependent RNA polymerase that recognizes and initiates synthesis of RNA on double-stranded DNA templates that carry the appropriate bacteriophage-specific promoter The polymerase is used in vitro to generate large quantities of RNA complementary to one strand of foreign DNA that has been cloned immediately downstream from the promoter in plasmids specifically designed for this purpose Vectors are available to synthesize RNA complementary to either strand of the template by changing the orientation of the promoter with respect to the cloned foreign DNA sequences (Butler and Chamberlin 1982; Melton et al 1984).

Bacteriophages T7 and T3 also synthesize DNA-dependent RNA polymerases that recognize and initiate synthesis of RNA on double-stranded DNA templates that carry the appropriate bacteriophage-specific promoter These polymerases are used in vitro just like the bacteriophage SP6 RNA polymerase Bacteriophages T7 and T3 RNA polymerases have been cloned and expressed in *E coli* (Davanloo et al 1984; Tabor and Richardson 1985; Morris et al 1986) and bacteriophage T7 RNA polymerase has been cloned and expressed in yeast (Chen et al 1987) This allows vectors carrying the bacteriophage T7 promoter to be used to express cloned genes in vivo

USES

1 Synthesis of single-stranded RNA for use as hybridization probes, functional mRNAs for in vitro translation systems, or substrates for in vitro splicing reactions Each of the three RNA polymerases has a high degree of specificity for its cognate promoter

2 The bacteriophage T7 transcription system has been used to express cloned genes in bacteria (Tabor and Richardson 1985, Studier and Moffatt 1986) and in yeast (Chen et al 1987)

Two types of bacteriophage T7 expression systems have been developed for *E coli* In the first system, stable lysogens are established with bacteriophage λ carrying the bacteriophage T7 RNA polymerase gene under the control of the *E coli lac*UV5 promoter Plasmids containing the gene of interest under the control of the bacteriophage T7 promoter are then introduced into the lysogens containing the bacteriophage T7 RNA polymerase gene Activation of the bacteriophage T7 promoter is then achieved by isopropylthio-β-D-galactoside induction of the *lac*UV5 promoter driving the bacteriophage T7 RNA polymerase gene In the second system, the bacteriophage T7 promoter/plasmid carrying the gene of interest is introduced into bacteria, and the bacteriophage T7 promoter is activated by infecting the bacteria with bacteriophage λ containing the bacteriophage T7 RNA polymerase gene

In yeast, the bacteriophage T7 RNA polymerase gene is placed under the control of a yeast promoter and stably introduced into yeast cells on an autonomously replicating vector. Expression is achieved by introducing into the yeast cells a second plasmid that contains the gene of interest under the control of the bacteriophage T7 promoter (Chen et al 1987).

Bacteriophages SP6, T3, and T7 DNA-dependent RNA Polymerases

Activity: 5′→3′ RNA polymerase

Substrate: Double-stranded DNA molecules containing bacteriophage SP6, T3, or T7 promoters

Reaction:

$$\text{double-stranded DNA} \xrightarrow[\substack{\text{Mg}^{++}\\ \text{rATP, rUTP, rGTP, rCTP}}]{\substack{\text{bacteriophage SP6, T3,}\\ \text{or T7 RNA polymerase}}} \text{RNA} + \text{PP}_i + \text{template}$$

For example:

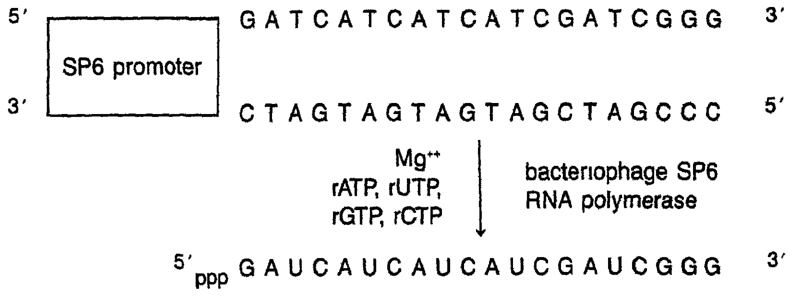


TABLE 5.9 *Properties of Bacteriophage T4 DNA Ligase and Polynucleotide Kinase*

	Bacteriophage T4 DNA ligase	Bacteriophage T4 polynucleotide kinase (forward reaction)	Bacteriophage T4 polynucleotide kinase (exchange reaction)
Nucleic acid substrate	cohesive ends—DNA blunt ends—DNA nicks RNA DNA hybrids	double-stranded DNA single-stranded RNA or DNA nick or gap oligonucleotide 3′ dNMP	double-stranded DNA single-stranded RNA or DNA nick or gap oligonucleotide
K_m	5′ cohesive termini, 0 6 μM blunt ends, 50 μM nicks, 1 5 pM	double-stranded DNA, 7 6 μM 5′ dNMP, 22–143 μM ATP, 14–140 μM[b]	ADP, 300 μM[a] ATP, 10 μM[a]
pH optimum	100% 7 5–8 0 40% 6 9 65% 8 3	7 4–8 0 (Tris · Cl) (phosphate buffer, 5% as active)	6 4 (imidazole)
Sulfhydryl requirement	+	+	+
Mg^{++} requirement	+	+	+
Effect of ionic strength	substrate-dependent	stimulated by NaCl and KCl excess KCl inhibits on all substrates except single-stranded DNA	no information
Inhibitors (50% inhibition)	>150 mM NaCl (activity measured at nicks)	$(NH_4)_2SO_4$, 7 mM P_i, 20 mM PP_i, 5 mM	P_i, 50 mM
Activators	10% PEG, intermolecular ligation[c] 150–200 mM NaCl, blunt-end ligation	polyamine, 2 mM, 300%	no information

[a] These are concentrations that give optimal activity at pH 6 4, not K_ms

[b] K_m for ATP varies with substrate

[c] Pheiffer and Zimmerman 1983, Hayashi et al 1986

LIGASES, KINASES, AND PHOSPHATASES

The enzymes used to join two pieces of DNA are called DNA ligases. The ligase used most often in cloning is encoded by bacteriophage T4, although there is a less versatile enzyme available from uninfected *E. coli* Both types of ligase are used primarily on DNA substrates with 5′-terminal phosphate groups

RNA ligase is a bacteriophage T4 enzyme that is capable of covalently joining single-stranded RNA (or DNA) molecules containing 5′-phosphate and 3′-hydroxyl termini However, the primary use of this enzyme has been in 3′ end-labeling of RNA This is accomplished using ^{32}P-labeled mononucleoside 3′,5′-bisphosphate (pNp), which is added to the 3′-hydroxyl terminus of RNA.

DNAs that lack the required phosphate residues can be prepared for ligation by phosphorylation with bacteriophage T4 polynucleotide kinase Conversely, DNAs can be rendered resistant to ligation by enzymatic removal of phosphate residues from their 5′ termini with phosphatases.

The properties of bacteriophage T4 DNA ligase and polynucleotide kinase are summarized in Table 5.9 and described in greater detail in the following pages The data in Table 5.9 have been collected from numerous papers published over the last 20 years. The values serve as accurate guidelines for using the enzymes, but optimal conditions will always vary slightly with the enzyme preparation (degree of purity) and the DNA preparation or when carrying out a sequence of enzymatic reactions (e g , digestion, end-filling, and ligation) in one mixture

Bacteriophage T4 DNA Ligase

(Bacteriophage T4-infected *E. coli*)

Bacteriophage T4 DNA ligase, a polypeptide of $M_r = 68,000$, catalyzes the formation of phosphodiester bonds between adjacent 3′-hydroxyl and 5′-phosphate termini in DNA (Weiss et al 1968). Its properties are summarized in Table 5 9

USES

1 Joining DNA molecules with compatible cohesive termini. Intermolecular ligation is stimulated by low concentrations of agents, such as polyethylene glycol (PEG), that promote the efficient interaction of macromolecules in aqueous solutions.

2. Joining blunt-ended double-stranded DNA molecules to one another or to synthetic linkers This reaction is much slower than ligation of cohesive termini However, the rate of blunt-end ligation is improved greatly by the addition of monovalent cations (150–200 mM NaCl) and low concentrations of PEG (Pheiffer and Zimmerman 1983, Hayashi et al 1986).

Notes

i. At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al 1968) One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into $[\gamma,\beta\text{-}^{32}P]$ATP in 20 minutes at 37°C One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs) 0 015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units

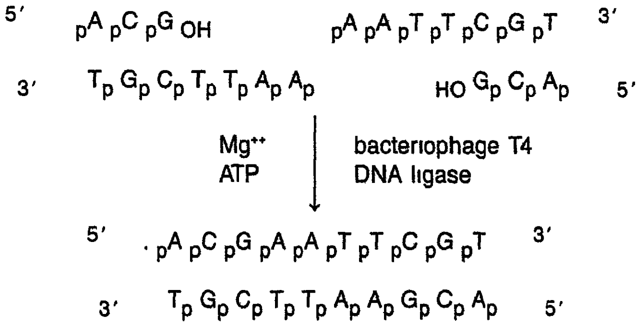
ii. Bacteriophage T4 DNA ligase is not inhibited by the presence of dNTPs and works adequately in virtually all buffers used for digestion of DNA with restriction enzymes

Bacteriophage T4 DNA Ligase

Activity: Ligation of cohesive DNA termini or nicks

Substrate: Active on double-stranded DNA with complementary cohesive termini that base pair to bring together 3′-hydroxyl and 5′-phosphate termini In addition, the enzyme is active on nicked DNA and active, albeit far less efficiently, on RNA substrates (For a more complete description of substrates, see Engler and Richardson 1982.)

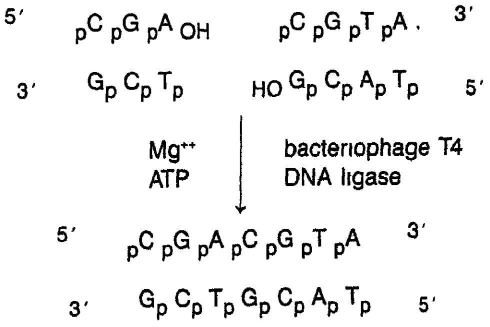
Reaction:


5′ pA pC pG OH pA pA pT pT pC pG pT 3′
3′ Tp Gp Cp Tp Tp Ap Ap HO Gp Cp Ap 5′
Mg++
ATP
bacteriophage T4
DNA ligase
5′ · pA pC pG pA pA pT pT pC pG pT 3′
3′ Tp Gp Cp Tp Tp Ap Ap Gp Cp Ap 5′

Activity: Ligation of blunt ends

Substrate: High concentrations of blunt-ended, double-stranded DNA containing 5′-phosphate and 3′-hydroxyl termini

Reaction:


5′ pC pG pA OH pC pG pT pA · 3′
3′ Gp Cp Tp HO Gp Cp Ap Tp 5′
Mg++
ATP
bacteriophage T4
DNA ligase
5′ pC pG pA pC pG pT pA 3′
3′ Gp Cp Tp Gp Cp Ap Tp 5′

E. coli DNA Ligase

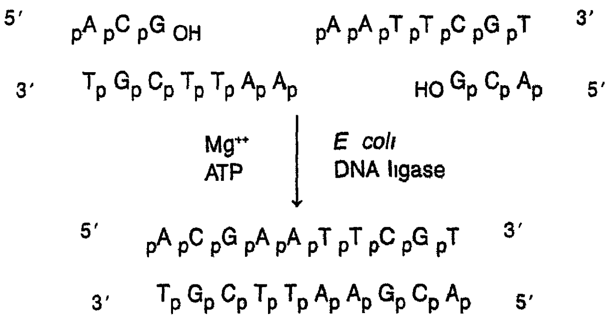
E. coli DNA ligase catalyzes the formation of phosphodiester bonds in double-stranded DNA containing complementary protruding 5′ or 3′ termini (Panasenko et al 1977, 1978). The reaction is catalyzed by NAD$^+$ Initial studies indicated that this enzyme would not ligate blunt-ended double-stranded DNA, but subsequent studies revealed that blunt-end ligation can be achieved in the presence of polyethylene glycol or Ficoll, compounds that act as volume excluders. This effectively increases the concentration of DNA termini and enzyme (Zimmerman and Pheiffer 1983). *E coli* DNA ligase has been used in cDNA cloning methods based on replacement synthesis, as described by Okayama and Berg (1982), because of its inability to join adjacent RNA and DNA segments that arise during the synthesis of the second strand of cDNA. It is not, however, widely used in other molecular cloning procedures, since bacteriophage T4 DNA ligase is capable of efficiently joining blunt ends in the absence of volume excluders *E coli* DNA ligase does not ligate RNA

E. coli DNA Ligase

Activity: Ligation of cohesive DNA termini or nicks

Substrate: Active on double-stranded DNA with complementary cohesive termini that base pair to bring together 3′-hydroxyl and 5′-phosphate termini In addition, the enzyme is active on nicked DNA

Reaction.


5′ pA pC pG OH pA pA pT pT pC pG pT 3′
3′ Tp Gp Cp Tp Tp Ap Ap HO Gp Cp Ap 5′
Mg++
ATP
E coli
DNA ligase
5′ pA pC pG pA pA pT pT pC pG pT 3′
3′ Tp Gp Cp Tp Tp Ap Ap Gp Cp Ap 5′

Bacteriophage T4 RNA Ligase

(Bacteriophage T4-infected *E. coli*)

Bacteriophage T4 RNA ligase catalyzes the covalent joining of 5′-phosphate single-stranded DNA or RNA to 3′-hydroxyl single-stranded DNA or RNA (Uhlenbeck and Gumport 1982).

USES

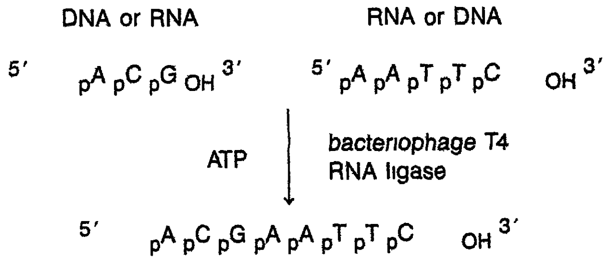
1. Because small molecules (e.g , pNp) are effective substrates, bacteriophage T4 RNA ligase can be used to radiolabel the 3′ termini of RNA molecules in vitro (Uhlenbeck and Gumport 1982)
2. Synthesis of oligodeoxyribonucleotides.
3. Bacteriophage T4 RNA ligase has been reported to stimulate the activity of bacteriophage T4 DNA ligase (Sugino et al 1977) However, agents such as polyethylene glycol that increase macromolecular crowding are equally effective and much less expensive.

Bacteriophage T4 RNA Ligase

Activity: RNA ligase

Substrate: 5′-phosphate acceptors include single-stranded DNA and RNA. Phosphate donors include single-stranded DNA and RNA and nucleotides such as pNp.

Reaction:



Bacteriophage T4 Polynucleotide Kinase

(Bacteriophage T4-infected *E. coli*)

Bacteriophage T4 polynucleotide kinase catalyzes the transfer of the γ-phosphate of ATP to a 5′ terminus of DNA or RNA (Richardson 1971). Two types of reactions are commonly used. In the *forward* reaction, the γ-phosphate is transferred to the 5′ terminus of dephosphorylated DNA (Richardson 1971). In the *exchange* reaction, an excess of ADP causes bacteriophage T4 polynucleotide kinase to transfer the terminal 5′ phosphate from phosphorylated DNA to ADP; the DNA is then rephosphorylated by transfer of a radiolabeled γ-phosphate from [γ-^{32}P]ATP (Berkner and Folk 1977). In addition to the phosphorylation activity of bacteriophage T4 polynucleotide kinase, the enzyme also contains a 3′ phosphatase activity (Richardson 1981). The properties of this enzyme are summarized in Table 5.9 and Richardson (1981).

USES

1. Radiolabeling 5′ termini in DNA for sequencing by the Maxam-Gilbert technique (Maxam and Gilbert 1977), for nuclease-S1 analysis, and for other uses requiring terminally labeled DNA.
2. Phosphorylating synthetic linkers and other fragments of DNA that lack terminal 5′ phosphates in preparation for ligation.

Notes

i. Bacteriophage T4 polynucleotide kinase is difficult to purify from infected cells, and impure preparations are not uncommon. Wherever possible, use bacteriophage T4 polynucleotide kinase that has been purified from cells expressing high levels of a cloned copy of the bacteriophage T4 gene.

ii. When setting up reactions involving the termini of nucleic acid molecules, the concentration of the reacting species can be calculated using the following table as a guide

Size of double-stranded DNA (in base pairs)	Amount of DNA required to contribute 1 pmole of 5′ termini (in μg)
50	1.7×10^{-2}
100	3.3×10^{-2}
250	8.4×10^{-2}
500	1.7×10^{-1}
1000	3.3×10^{-1}
2500	8.4×10^{-1}
5000	1.7

iii. Spermidine stimulates incorporation of [γ-^{32}P]ATP and inhibits a nuclease present in some preparations of bacteriophage T4 polynucleotide kinase.

iv. ATP should be present at a concentration of at least 1 μM in the forward reaction and at least 2 μM in the exchange reaction, but optimal activity occurs at higher concentrations (see Table 5 9)

v. The dephosphorylated DNA should be rigorously purified by gel electrophoresis, density gradient centrifugation, or chromatography on columns of Sepharose CL-4B in order to remove low-molecular-weight nucleic acids Although such contaminants may make up only a small fraction of the weight of the nucleic acids in the preparation, they provide a much larger proportion of the 5′ termini Unless steps are taken to remove them, contaminating low-molecular-weight DNAs and RNAs can be the predominant species of nucleic acids that are labeled in bacteriophage T4 polynucleotide kinase reactions.

vi. Ammonium ions are strong inhibitors of bacteriophage T4 polynucleotide kinase Therefore, DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with the kinase

vii. Low concentrations of phosphate also inhibit bacteriophage T4 polynucleotide kinase, imidazole buffer (pH 6 4) is therefore the buffer of choice for the exchange reaction, and Tris buffer is the buffer of choice for the forward reaction

Bacteriophage T4 Polynucleotide Kinase

Activity: Kinase (forward reaction)

Substrate: Single- or double-stranded DNA with 5′-hydroxyl terminus; RNA with a 5′-hydroxyl terminus The enzyme phosphorylates protruding 5′ single-stranded termini more rapidly than blunt ends or recessed 5′ termini, however, with sufficient enzyme and ATP, such termini can be completely phosphorylated The reaction at nicks or gaps in double-stranded DNA is less efficient than for single-stranded termini; however, with sufficient concentrations of ATP and enzyme, gaps can be completely phosphorylated and nicks can be phosphorylated to 70%

Reaction:

$$\mathrm{DNA}_{\mathrm{OH}}{}^{5'} \text{ or } \mathrm{RNA}_{\mathrm{OH}}{}^{5'} \xrightarrow[\substack{[\gamma\text{-}^{32}\mathrm{P}]\mathrm{ATP} \\ \text{dithiothreitol} \\ \mathrm{Mg}^{++}}]{\substack{\text{bacteriophage T4} \\ \text{polynucleotide kinase}}} \substack{5'[^{32}\mathrm{P}]\mathrm{DNA} \text{ or } 5'[^{32}\mathrm{P}]\mathrm{RNA} \\ + \text{ ADP}}$$

For example:

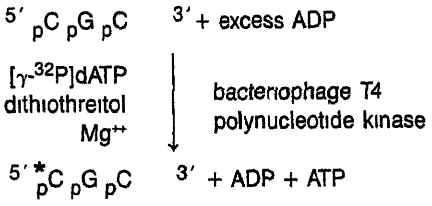
$$\begin{tikzcd} 5'\ {}_{\mathrm{HO}}C\ {}_{p}G\ {}_{p}C\quad 3' \arrow[d, "\substack{\text{bacteriophage T4} \\ \text{polynucleotide kinase}}", "\substack{[\gamma\text{-}^{32}\mathrm{P}]\mathrm{dATP} \\ \text{dithiothreitol} \\ \mathrm{Mg}^{++}}"'] \\ 5'\ {}_{\overset{*}{p}}C\ {}_{p}G\ {}_{p}C\quad 3' \\ + \text{ ADP} \end{tikzcd}$$

Bacteriophage T4 Polynucleotide Kinase

Activity: Kinase (exchange reaction)

Substrate: Single-stranded DNA with a 5′-phosphate terminus is most efficiently labeled (96%). Recessed 5′-phosphate termini are labeled to 70% with sufficient enzyme 5′-phosphate groups at nicks are labeled 30-fold less efficiently than single-stranded 5′-phosphate termini

Reaction:



$$5'\;{}_{p}C\;{}_{p}G\;{}_{p}C\quad 3' + \text{excess ADP}$$

$$\left.\begin{array}{r}[\gamma\text{-}^{32}\text{P}]\text{dATP}\\ \text{dithiothreitol}\\ \text{Mg}^{++}\end{array}\right\downarrow\;\begin{array}{l}\text{bacteriophage T4}\\ \text{polynucleotide kinase}\end{array}$$

$$5'\;{}^{*}_{p}C\;{}_{p}G\;{}_{p}C\quad 3' + \text{ADP} + \text{ATP}$$

Alkaline Phosphatases

(*E. coli* and calf intestine)

Both bacterial alkaline phosphatase (BAP) and calf intestinal alkaline phosphatase (CIP) catalyze the removal of 5′-phosphate residues from DNA, RNA, rNTPs, and dNTPs

USES

1. Removing 5′ phosphates from DNA or RNA prior to labeling 5′ termini with ^{32}P
2. Removing 5′ phosphates from fragments of DNA to prevent self-ligation

Note

BAP is the more active of the two alkaline phosphatases, but it is also far more resistant to heat and detergents. It is therefore difficult to inhibit BAP completely at the end of dephosphorylation reactions. Proteinase K is used to digest CIP, which must be completely removed if subsequent ligations are to work efficiently An alternative method is to inactivate the CIP by heating to 65°C for 1 hour (or 75°C for 10 minutes) in the presence of 5 mM EDTA (pH 8.0) and then to purify the dephosphorylated DNA by extraction with phenol chloroform

Alkaline Phosphatases

Activity: Phosphatase

Substrate: Single- or double-stranded DNA and RNA, rNTPs and dNTPs.

Reaction:

$$5'_{\mathrm{p}}\mathrm{DNA} \text{ or } 5'_{\mathrm{p}}\mathrm{RNA} \xrightarrow{\text{alkaline phosphatase}} 5'_{\mathrm{OH}}\mathrm{DNA} \text{ or } 5'_{\mathrm{OH}}\mathrm{RNA}$$

NUCLEASES

Nuclease BAL 31

(*Alteromonas espejiana* BAL 31)

BAL 31 is predominantly a 3′ exonuclease that removes mononucleotides from both 3′ termini of the two strands of linear DNA. BAL 31 is also an endonuclease; thus, the single-stranded DNA generated by the 3′ exonuclease activity is degraded by the endonuclease The mechanisms of these reactions are complex and are summarized below

Degradation is absolutely dependent on the presence of calcium, and the reaction can therefore be stopped at different stages by the addition of the chelating agent EGTA Because degradation occurs relatively uniformly from the termini of DNA, digestion with BAL 31 can be used to map restriction sites in small fragments of DNA (Legerski et al 1978) DNA is digested with BAL 31, and samples are withdrawn at different times and placed in a solution containing EGTA After digestion of these samples with the restriction enzyme of interest, restriction fragments can be seen to disappear in a defined order By using a DNA consisting of vector sequences at one terminus (for which the restriction map is known) and unmapped sequences at the other, it is possible to distinguish fragments from the two termini and to deduce the order of the fragments in the unmapped DNA

BAL 31 can also be used to remove unwanted sequences from the termini of DNAs before cloning After treatment with the exonuclease/endonuclease, the termini of the DNA are repaired with bacteriophage T4 DNA polymerase or the Klenow fragment of *E coli* DNA polymerase I Synthetic linkers are added to the DNA, which is then inserted into a suitable vector In this way, it is possible to generate a set of deletions from a defined endpoint in DNA

Although the enzyme is predominantly a 3′ exonuclease, it also has a DNA endonuclease activity and cleaves internally in single-stranded regions of DNA or in double-stranded DNA that contains helical distortions (Lau and Gray 1979, Gray et al 1981, Wei et al 1983). BAL 31 will also digest RNA, albeit inefficiently

USES

1 Removing nucleotides from the termini of double-stranded DNA in a controlled manner The shortened molecules can be used for a variety of purposes such as to produce deletions, to position a desired sequence next to a promoter or other controlling element, or to attach synthetic linkers at desired sites in the DNA

2 Mapping restriction sites in DNA (Legerski et al. 1978)

3 Mapping secondary structure in DNA, for example, junctions between B- and Z-DNA or sites of covalent or noncovalent modifications in double-stranded DNA (Gray et al 1981, Wei et al 1983).

4 Removing nucleotides from double-stranded RNA in preparing recombinant RNAs (Miele et al. 1983).

Notes

i. The rate at which BAL 31 removes nucleotides from the 5′ and 3′ termini of double-stranded DNA is given by the equation:

$$\frac{dM_t}{dt} = \frac{-2V_m M_n}{[K_m + (S)_0]}$$

where M_t = molecular weight of linear double-stranded DNA after t minutes of digestion, V_m = maximum reaction velocity (expressed as moles of nucleotide removed/liter/minute), M_n = average molecular mass of sodium mononucleotide (taken as 330 daltons), K_m = Michaelis-Menten constant (expressed as moles of double-stranded DNA termini/liter), and $(S)_0$ = moles of double-stranded DNA termini/liter at $t = 0$ minutes (remains constant until a significant portion of the molecules have undergone complete degradation).

At an enzyme concentration of 40 units/ml:

$V_m = 2.4 \times 10^{-5}$ moles of nucleotide removed/liter/minute
$K_m = 4.9 \times 10^{-9}$ moles of double-stranded DNA termini/liter

Assuming that the value of V_m alters in proportion to enzyme dilution (5/40 in the example below where the enzyme concentration is 5 units/ml), then the rate of degradation of a 2-kb fragment of DNA in a 100-μl reaction containing 10 μg of DNA and 0.5 unit of enzyme will be

$$= \frac{-2(5/40)(2.4 \times 10^{-5})(330)}{(4.9 \times 10^{-9}) + (1.5 \times 10^{-7})}$$

$$= \frac{-(1/4)(2.4 \times 10^{-5})(330)}{1.5049 \times 10^{-7}}$$

$= -1.31 \times 10^{4}$ daltons/minute/DNA molecule
$= -19$ bp/minute/DNA molecule
$= -9.5$ bp/minute/terminus of double-stranded DNA

In most cases (i.e., when digesting DNAs less than 10 kb in length and when the concentration of DNA in the reaction exceeds 20 μg/ml), the term for K_m in the denominator of the equation becomes insignificant.

ii. When the products of BAL 31 digestion are to be ligated, it is important to consider that the 3′ exonuclease activity of the enzyme works about 20-fold more efficiently than the DNA endonuclease. Thus, the average length of single-stranded tails created by digestion of linear double-stranded DNA is dependent on the enzyme concentration. At high enzyme concentrations (2–5 units/ml), an average of five nucleotides of single-stranded DNA remain per terminus and 10–20% of the molecules can be ligated to blunt-ended DNA without further treatment. At low enzyme concentrations (0.1–0.2 unit/ml), the single-stranded termini may be very long and the efficiency of blunt-end ligation is very low. Repair with bacteriophage T4 DNA polymerase (or, in some cases, the Klenow fragment of *E. coli* DNA polymerase I) is almost obligatory at either high or low concentrations of BAL 31.

iii. Most commercial preparations of BAL 31 contain two kinetically distinct forms of the enzyme, a fast and a slow form. The slow form is a proteolytic degradation product of the fast form. Pure preparations of the fast form are available, but they are expensive (Wei et al. 1983). Results using mixed preparations will vary, depending on the relative amounts of the two forms in the initial preparation and the rate of conversion of the fast to the slow form during the assay.

Preparations rich in the fast form are preferred for such tasks as removal of long (> 1000 bp) segments from the termini of double-stranded DNA, degradation of double-stranded RNA; and mapping of restriction sites, B-Z DNA junctions, and lesions in double-stranded DNA. The slow form of the enzyme is used to remove short segments (10–100 bp) from the termini of double-stranded DNA Mixed preparations of the enzyme can be used for any of these tasks, although the results will vary as mentioned above.

iv. BAL 31 works asynchronously, generating a population of DNA molecules whose termini have been resected to various extents and whose single-stranded tails vary in length. Following digestion with BAL 31 and repair with bacteriophage T4 DNA polymerase (or the Klenow fragment of *E. coli* DNA polymerase I), it is often more efficient to isolate DNAs of the required size by gel electrophoresis rather than to screen very large numbers of randomly generated clones

v BAL 31 degrades AT-rich sequences significantly more rapidly than it degrades GC-rich regions Thus, molecules that terminate in AT-rich regions are underrepresented in populations of DNAs that have been digested with the enzyme

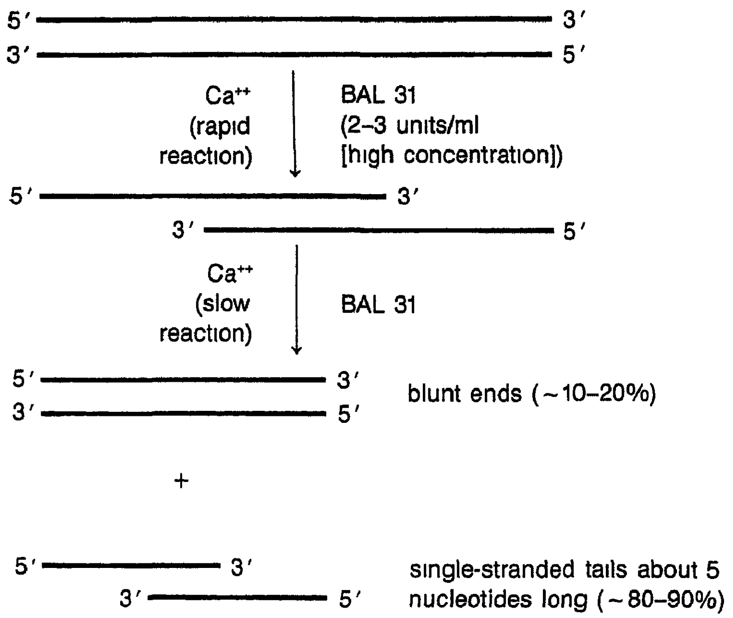
vi. BAL 31 should not be frozen Store the enzyme at 4°C.

Nuclease BAL 31

Activity: Exonuclease/endonuclease

Substrate: BAL 31 degrades double-stranded DNA sequentially from both termini. The mechanism is thought to involve a rapid exonucleolytic degradation followed by a slow endonucleolytic reaction on the complementary strand. Double-stranded DNA with blunt or protruding 3′-hydroxyl termini are degraded to shorter double-stranded molecules. The enzyme is also active at nicks, on single-stranded DNA with 3′-hydroxyl termini, and on double-stranded RNA molecules.

Reaction:

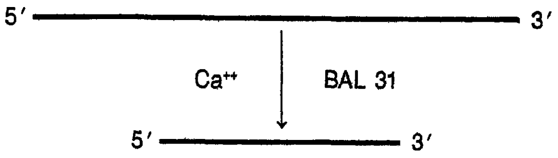


Nuclease BAL 31

Activity: Exonuclease (shortens single-stranded DNA)

Substrate: Single-stranded DNA with 3′-hydroxyl termini.

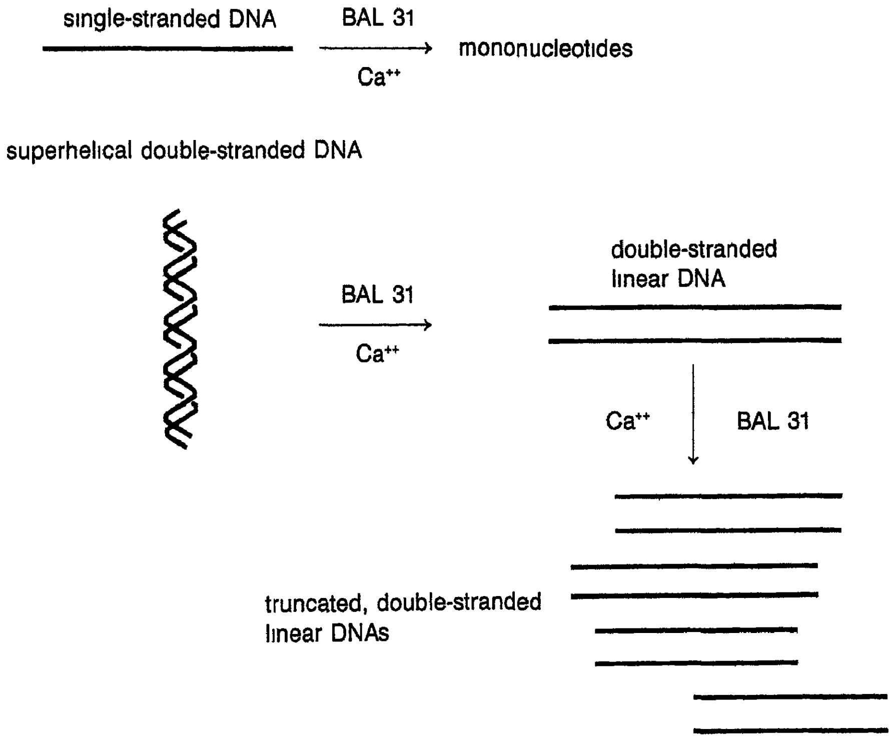
Reaction:



Activity: Endonuclease

Substrate: Single-stranded DNA, supercoiled DNA, DNA with B-DNA, Z-DNA junctions and other non-B-DNA conformations

Reaction:



Nuclease S1

(Aspergillus oryzae)

Nuclease S1 degrades single-stranded DNA or RNA (Vogt 1973) to yield 5′-phosphate mono- or oligonucleotides. Double-stranded DNA, double-stranded RNA, and DNA·RNA hybrids are relatively resistant to the enzyme However, double-stranded nucleic acids are digested completely by nuclease S1 if they are exposed to very large amounts of the enzyme Moderate amounts of the enzyme will cleave double-stranded nucleic acids at nicks or small gaps (Kroeker and Kowalski 1978).

USES

1. Analyzing the structure of DNA:RNA hybrids (Berk and Sharp 1977; Favaloro et al. 1980)
2 Removing single-stranded tails from DNA fragments to produce blunt ends.
3. Opening the hairpin loop generated during synthesis of double-stranded cDNA.

Note

Since the enzyme works at low pH, depurination often occurs, which limits the usefulness of nuclease S1 for some applications

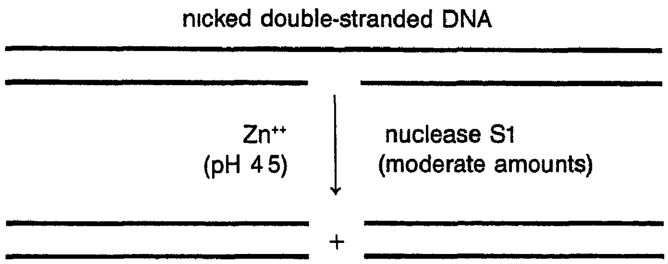
Nuclease S1

Activity: Single-strand-specific nuclease

Substrate: Single-stranded DNA or RNA, more active on DNA than on RNA

Reaction:

$$\text{single-stranded DNA or RNA} \xrightarrow[\substack{\text{Zn}^{++} \\ \text{(pH 4.5)}}]{\text{nuclease S1}} {}^{5'}{}_{\text{p}}\text{dN or } {}^{5'}{}_{\text{p}}\text{rN}$$



Mung-bean Nuclease

(Mung-bean sprouts)

Mung-bean nuclease degrades single-stranded DNA to mono- or oligonucleotides with phosphate groups at their 5′ termini (Laskowski 1980). Double-stranded DNA, double-stranded RNA, and DNA:RNA hybrids are relatively resistant to the enzyme. However, double-stranded nucleic acids are digested completely by mung-bean nuclease if they are exposed to very large amounts of the enzyme (Kroeker and Kowalski 1978)

Although mung-bean nuclease and nuclease S1 are similar to each other in their physical and catalytic properties, mung-bean nuclease may be less severe in its action than nuclease S1. For example, nuclease S1 has been shown to cleave the DNA strand opposite a nick in a duplex, whereas mung-bean nuclease will only attack the nick after it has been enlarged to a gap several nucleotides in length (Kroeker and Kowalski 1978)

USE

Converting protruding termini of DNA to blunt ends

Ribonuclease A

(Bovine pancreas)

RNAase A is an endoribonuclease that specifically attacks single-stranded RNA 3′ to pyrimidine residues and cleaves the phosphate linkage to the adjacent nucleotide The end products are pyrimidine 3′ phosphates and oligonucleotides with terminal pyrimidine 3′ phosphates (Davidson 1972). RNAase A, which works in the absence of cofactors and divalent cations, can be inhibited by placental RNAase inhibitor (Blackburn et al 1977) or by vanadyl-ribonucleoside complexes (Puskas et al 1982).

$$5'\ {}_pA_pG_pG_pC_p\overset{\downarrow}{}C_p\overset{\downarrow}{}G_pA_pA_pG_pU_p\overset{\downarrow}{}G_pC_p\overset{\downarrow}{}A_pG_pG\ 3'$$

$$\Big\downarrow\ \text{RNAase A}$$

$$5'\ {}_pA_pG_pG_pC_p + C_p + G_pA_pA_pG_pU_p + G_pC_p + A_pG_pG\ 3'$$

USES

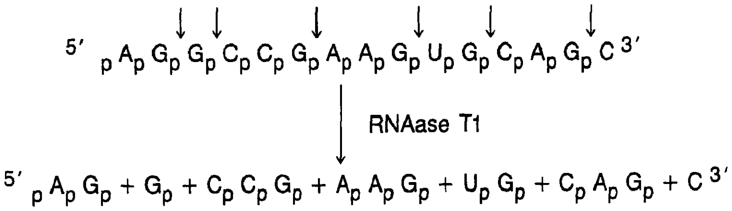
1 Removing unhybridized regions of RNA from DNA·RNA hybrids.

2 Mapping single-base mutations in DNA or RNA (Myers et al 1985; Winter et al 1985) In this method, single-base mismatches in RNA·DNA or RNA.RNA hybrids are recognized and cleaved by RNAase A A ^{32}P-labeled RNA probe complementary to wild-type DNA or RNA is synthesized in vitro using a plasmid containing a bacteriophage SP6 or T7 promoter The RNA probe is then annealed to test DNA or RNA containing a single-base substitution The resulting single-base mismatch is cleaved by RNAase A, and the location of the mismatch is then determined by analyzing the sizes of the cleavage products by gel electrophoresis Approximately 50% of all possible single-base mismatches can be detected by this method

Ribonuclease T1

(*Aspergillus oryzae*)

RNAase T1 is an endoribonuclease that specifically attacks the 3′-phosphate groups of guanine nucleotides and cleaves the 5′-phosphate linkage to the adjacent nucleotide. The end products are guanosine 3′ phosphates and oligonucleotides with terminal guanosine-3′-phosphate groups (Davidson 1972).


5′ pApGpGpCpCpGpApApGpUpGpCpApGpC 3′
RNAase T1
5′ pApGp + Gp + CpCpGp + ApApGp + UpGp + CpApGp + C 3′

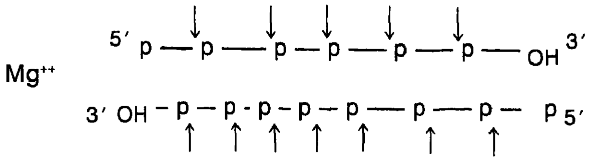
USE

Removing unhybridized regions of RNA from DNA RNA hybrids.

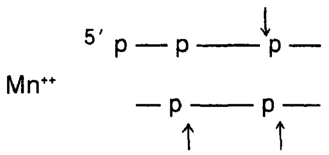
Deoxyribonuclease I

(Bovine pancreas)

DNAase I is an endonuclease that hydrolyzes double-stranded or single-stranded DNA preferentially at sites adjacent to pyrimidine nucleotides. Products are a complex mixture of 5′-phosphate mono- and oligonucleotides. In the presence of Mg^{++}, DNAase I attacks each strand of DNA independently, and the sites of cleavage are distributed in a statistically random fashion.


Mg++
5′ p — p — p — p — p — p — OH 3′
3′ OH – p – p · p – p – p — p — p — p 5′

In the presence of Mn^{++}, DNAase I cleaves both strands of DNA at approximately the same site (Melgar and Goldthwait 1968) to yield fragments of DNA that are blunt-ended or that have protruding termini only one or two nucleotides in length



USES

1 Introducing random nicks into double-stranded DNAs in preparation for radiolabeling by nick translation (see Chapter 10)

2 Introducing a single nick into closed circular DNAs in preparation for resection prior to bisulfite-mediated mutagenesis (Greenfield et al 1975).

3 Generating random clones for sequencing in bacteriophage M13 vectors (Anderson 1981)

4 Analysis of protein.DNA complexes (DNAase footprinting) (Galas and Schmitz 1978)

Exonuclease III

(E. coli)

Exonuclease III catalyzes the stepwise removal of 5′ mononucleotides from the 3′-hydroxyl termini of double-stranded DNA (Weiss 1976) Linear double-stranded DNA and circular DNAs containing nicks or gaps are substrates. The activity of the enzyme results in the formation of long single-stranded regions in double-stranded DNA. The enzyme also carries three other activities. an endonuclease specific for apurinic DNA, an RNAase H activity (Rogers and Weiss 1980), and a 3′ phosphatase activity, which removes 3′-phosphate termini but does not cleave internal phosphodiester bonds The exonuclease will not degrade single-stranded DNA or double-stranded DNA with a protruding 3′ terminus (Rogers and Weiss 1980)

Exonuclease III is nonprocessive and typically generates populations of molecules that have been resected to similar extents. This property simplifies the task of isolating DNA molecules whose lengths have been reduced by the desired amount.

USES

1 Generating partially resected DNAs that can be used as substrates for the Klenow fragment of *E coli* DNA polymerase I (e g , in the preparation of strand-specific probes) (See Figure 5.3 for a similar application for bacteriophage T4 DNA polymerase)

2 Generating nested sets of deletions of the terminal sequences of double-stranded linear DNAs This reaction is usually carried out in conjunction with mung-bean nuclease or nuclease S1 and is an alternative to using BAL 31 Because exonuclease III will degrade DNA with recessed 3′ termini but not termini with protruding 3′ single strands, it can be used to create unidirectional sets of deletions (Henikoff 1984) Thus, if the substrate molecule carries a protruding 3′ terminus at one end (e.g , created by digestion with *Pst*I) and a recessed 3′ terminus at the other, exonuclease III will digest only in one direction (from the recessed 3′ terminus) After removal of the resulting single-stranded segments (with nuclease S1 or mung-bean nuclease) and repair with bacteriophage T4 DNA polymerase, the deleted molecules can be inserted into an appropriate vector by blunt-end ligation

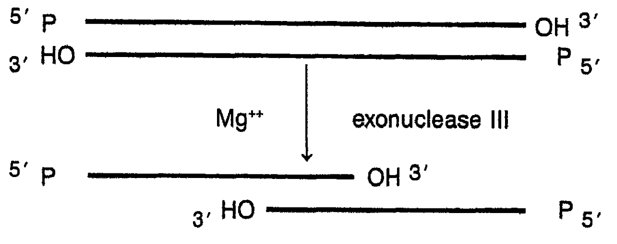
3. Some methods of site-specific mutagenesis use thiophosphate derivatives of the dNTPs for second-strand synthesis primed by the mutagenic primer The parental template strand can be preferentially degraded with exonuclease III, increasing the frequency of mutants obtained upon transformation of *E coli*, since exonuclease III will not cleave thioester bonds.

Exonuclease III

Activity: 3′ Exonuclease

Substrate: This enzyme is active on 3′-hydroxyl termini of double-stranded DNA with blunt ends or with ends containing unpaired 5′ termini and recessed 3′ termini. 3′-hydroxyl termini at nicks in double-stranded DNA are also substrates The DNA must contain phosphodiester bonds, thioesters are not cleaved

Reaction:



$+\ {}^{5'}{}_{p}N_{OH}$

For example.

$$5'\quad {}_{p}C\;{}_{p}G\;{}_{p}A\;{}_{p}T\;{}_{p}T\;{}_{p}A\;{}_{p}G\;{}_{p}C_{OH}\quad 3'$$

$$3'\quad G_{p}\;C_{p}\;T_{p}\;A_{p}\;A_{p}\;T_{p}\;C_{p}\;G_{p}\quad 5'$$

$$\mathrm{Mg^{++}}\ \Big\downarrow\ \text{exonuclease III}$$

$$5'\quad {}_{p}C_{OH}\quad 3'$$

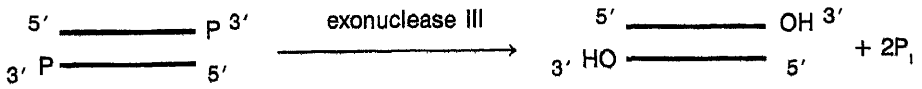
$$3'\quad G_{p}\;C_{p}\;T_{p}\;A_{p}\;A_{p}\;T_{p}\;C_{p}\;G_{p}\quad 5'$$

$$+\ {}^{5'}{}_{p}A_{OH} + {}^{5'}{}_{p}T_{OH} + {}^{5'}{}_{p}G_{OH} + {}^{5'}{}_{p}C_{OH}$$

Activity: 3′ Phosphatase

Substrate: Double- or single-stranded DNA with a 3′-phosphate terminus, internal phosphodiester bonds are not cleaved

Reaction:



Bacteriophage λ Exonuclease

(Bacteriophage λ-infected *E. coli*)

Bacteriophage λ exonuclease catalyzes the processive, stepwise release of 5′ mononucleotides from double-stranded DNA. Although the preferred substrate is double-stranded DNA with a terminal 5′ phosphate (Little et al 1967), the enzyme will also work, albeit 100-fold less efficiently, on single-stranded DNA. Double-stranded DNAs with nicks or gaps will not serve as substrates.

USES

Bacteriophage λ exonuclease was used for a wide variety of purposes in the early days of molecular cloning (for review, see Little 1981). Today, it is used chiefly to modify the 5′-phosphate termini of DNAs that are to be used as substrates for other enzymes (e.g., terminal transferase)

Bacteriophage λ Exonuclease

Activity: 5′ Exonuclease

Substrate: Double-stranded DNA with terminal 5′ phosphate or with protruding 5′ termini.

Reaction:

$$\begin{array}{l} 5' \cdot {}_{p}C\,{}_{p}G\,{}_{p}A\,{}_{p}T\,{}_{p}G\,{}_{p}G\,{}_{p}C\,{}_{p}T\,{}_{p}A\,{}_{p}C\,{}_{OH}\ 3' \\ 3' \quad G_p\,C_p\,T_p\,A_p\,C_p\,C_p\,G_p\,A_p\,T_p\,G_p\ 5' \end{array}$$

$$\text{Mg}^{++}\ \Big\downarrow\ \begin{array}{l}\text{bacteriophage } \lambda \\ \text{exonuclease}\end{array}$$

$$\begin{array}{l} 5' \cdot {}_{p}C\,{}_{p}G\,{}_{p}A\,{}_{p}T\,{}_{p}G\,{}_{p}G\,{}_{p}C\,{}_{p}T\,{}_{p}A\,{}_{p}C\,{}_{OH}\ 3' \\ 3' \cdot G_p\,C_p\,T_p\,A_p\ 5' \\ +\ {}^{5'}{}_{p}G_{OH} + {}^{5'}{}_{p}C_{OH} + {}^{5'}{}_{p}T_{OH} + {}^{5'}{}_{p}A_{OH} \end{array}$$

DNA-binding Proteins

Single-stranded DNA-binding Protein (SSB)

(*E. coli* or bacteriophage T4-infected *E. coli*)

SSBs bind cooperatively to single-stranded DNA, but not to double-stranded DNA Their usefulness as reagents in molecular cloning derives from their ability to destabilize intrastrand secondary structures; they accelerate reannealing of complementary polynucleotides and increase the processivity of DNA polymerases by removing intrastrand secondary structures that form barriers to the progression of these enzymes (for review, see Chase and Williams 1986) Because of this property, SSBs have become useful reagents in DNA sequencing Two well-studied SSBs are those encoded by *E coli* and by gene *32* of bacteriophage T4 (known as the gene *32* protein)

USES

1 Visualizing DNA by electron microscopy (Griffith et al 1984)

2 Site-directed mutagenesis involving D-loops (Shortle et al 1980) (in conjunction with RecA protein [see page 5.88]).

3 Increasing the chain length of the product in any reaction catalyzed by a DNA polymerase on long templates. Particular SSBs may stimulate specific DNA polymerases (see Kowalczykowski et al 1981) used in DNA sequencing reactions.

RecA Protein

(E. coli)

This protein ($M_r = 37,842$) is encoded by the *recA* gene of *E. coli*. It has several useful activities associated with its role in recombination in the cell. It binds single-stranded DNA, promotes uptake of homologous single-stranded DNA into double-stranded DNA, and is a DNA-dependent ATPase.

USES

1. Analysis of DNA structure by electron microscopy (Wasserman and Cozzarelli 1985)
2. In vitro mutagenesis using D-looping (Shortle et al. 1980) (see Figure 5.5)
3. Selection of specific sequences from libraries of double-stranded DNA via the formation of a D-loop (Griffith and Shores 1985, Rigas et al. 1986)

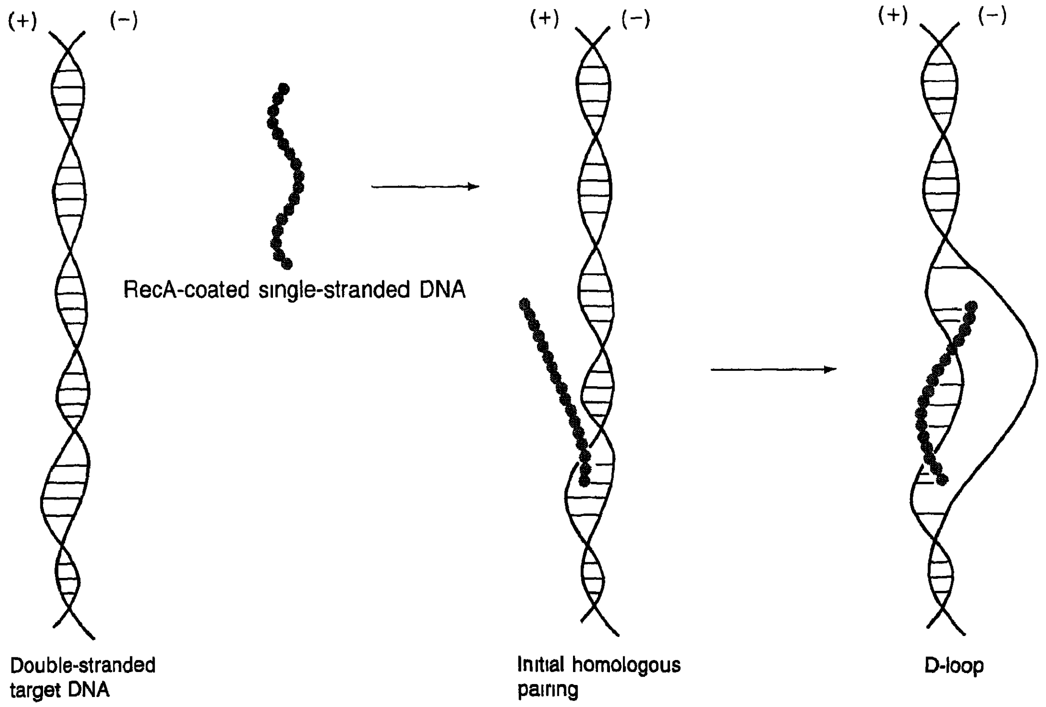

(+)
(−)
RecA-coated single-stranded DNA
(+)
(−)
(+)
(−)
Double-stranded target DNA
Initial homologous pairing
D-loop

FIGURE 5.5
The use of RecA protein to produce a displacement loop in double-stranded DNA

Topoisomerase I

(Calf thymus)

Topoisomerase I catalyzes the removal of superhelical turns from covalently closed circular double-stranded DNA by transient breakage and rejoining of phosphodiester bonds. Topoisomerase I is relatively insensitive to the superhelical density of the DNA and is active in solutions containing EDTA In contrast to prokaryotic type I topoisomerases, the calf thymus enzyme relaxes both positively and negatively supercoiled DNA to completion.

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6

Gel Electrophoresis of DNA

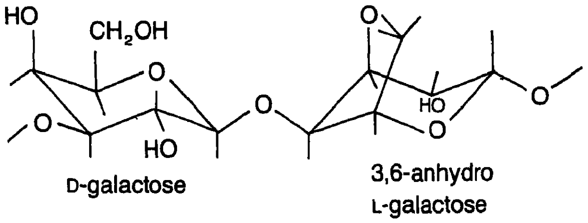
Electrophoresis through agarose or polyacrylamide gels is the standard method used to separate, identify, and purify DNA fragments (electrophoresis of RNA is discussed in Chapter 7) The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1–10 ng of DNA can be detected by direct examination of the gel in ultraviolet light (Sharp et al 1973). If necessary, these bands of DNA can be recovered from the gel and used for a variety of cloning purposes.

Agarose and polyacrylamide gels can be poured in a variety of shapes, sizes, and porosities and can be run in a number of different configurations The choices within these parameters depend primarily on the sizes of the fragments being separated. Polyacrylamide gels are most effective for separating small fragments of DNA (5–500 bp) Their resolving power is extremely high, and fragments of DNA that differ in size by as little as 1 bp can be separated from one another. Although they can be run very rapidly and can accommodate comparatively large quantities of DNA, polyacrylamide gels have the disadvantage of being more difficult to prepare and handle than agarose gels Polyacrylamide gels are run in a vertical configuration in a constant electric field

Agarose gels have a lower resolving power than polyacrylamide gels but have a greater range of separation DNAs from 200 bp to approximately 50 kb in length can be separated on agarose gels of various concentrations Agarose gels are usually run in a horizontal configuration in an electric field of constant strength and direction Larger DNAs, up to 10,000 kb in length, can be separated by pulsed-field gel electrophoresis, in which the direction of the electric flux is changed periodically Each of these types of gel electrophoresis will be discussed in detail in this chapter

Agarose Gel Electrophoresis

Agarose, which is extracted from seaweed, is a linear polymer whose basic structure is



Commercially available agarose is not completely pure; it is contaminated with other polysaccharides, salts, and proteins. The amount of contamination varies from batch to batch of agarose and from manufacturer to manufacturer These differences can affect both the migration of the DNA and the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions Because of the great increase in demand during the past 10 years, most manufacturers now prepare special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide

Some manufacturers also sell chemically modified forms of agarose that gel and melt at low temperature without significant deterioration in the strength of the hardened gel Such chemically modified agaroses are used chiefly for preparative electrophoresis of DNA and for digestion of DNA with restriction enzymes in situ Special grades of low-gelling-temperature agarose that can be used to analyze very small fragments of DNA (10–500 bp) are also available from some manufacturers Gels made with agarose of this type have a greater resolving power than gels made with normal agarose, but the resolution obtained from these gels still cannot compare with the resolution obtained from polyacrylamide gels Furthermore, since these gels contain a high concentration of agarose (4–10%), DNA fragments eluted from the gel are frequently contaminated with inhibitors that prevent further enzymatic manipulation

Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode The rate of migration is determined by a number of parameters, which are discussed on the following pages

Factors Affecting the Rate of DNA Migration in Agarose Gels

MOLECULAR SIZE OF THE DNA

Molecules of linear double-stranded DNA, which tend to become oriented in an electric field in an end-on position (Fisher and Dingman 1971, Aaij and Borst 1972), migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs (Helling et al 1974) (Figure 6.1) Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules

AGAROSE CONCENTRATION

A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose There is a linear relation-

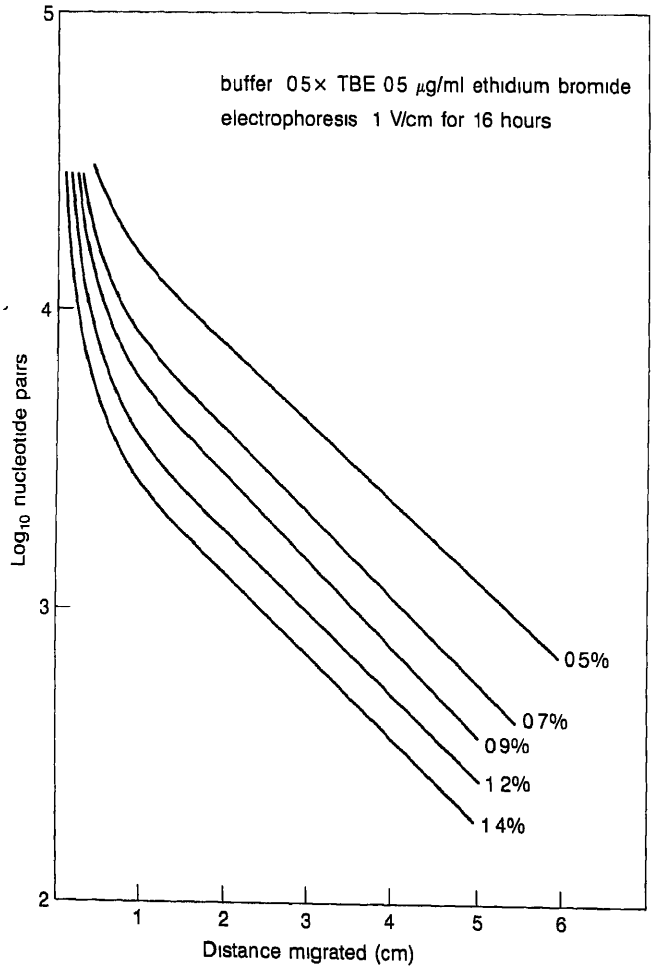


FIGURE 6.1
The relationship between the size of DNA and its electrophoretic mobility

TABLE 6.1 Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5–60
0.6	1–20
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

ship between the logarithm of the electrophoretic mobility of DNA (μ) and the gel concentration (τ), which is described by the equation:

$$\log \mu = \log \mu_0 - K_r \tau$$

where μ_0 is the free electrophoretic mobility of DNA and K_r is the retardation coefficient, a constant that is related to the properties of the gel and the size and shape of the migrating molecules. Thus, by using gels of different concentrations, it is possible to resolve a wide size range of DNA molecules (see Table 6.1).

CONFORMATION OF THE DNA

Superhelical circular (form I), nicked circular (form II), and linear (form III) DNAs of the same molecular weight migrate through agarose gels at different rates (Thorne 1966, 1967). The relative mobilities of the three forms depend primarily on the agarose concentration in the gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the form I DNA (Johnson and Grossman 1977). Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed.

An unambiguous method for identifying the different conformational forms of DNA is to carry out electrophoresis in the presence of increasing quantities of ethidium bromide. As the concentration of ethidium bromide increases, more of the dye becomes bound to the DNA. The negative superhelical turns in form I molecules are progressively removed, the radii of the molecules increase, and their rate of migration decreases. At the critical free-dye concentration, where no superhelical turns remain, the rate of migration of form I DNA reaches its minimum value. As still more ethidium bromide is added, positive superhelical turns are generated, the DNA molecules become more compact, and their mobility increases rapidly. Simultaneously, the mobilities of form II and form III DNA decrease differentially due to charge neutralization and the greater stiffness imparted to the DNA by the ethidium bromide. For most preparations of form I DNA, the critical concentration of free ethidium bromide is in the range of 0.1 μg/ml to 0.5 μg/ml.

APPLIED VOLTAGE

At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA increases differentially. Thus, the effective range of separation in agarose gels decreases as the voltage is increased. To obtain maximum resolution of DNA fragments greater than 2 kb in size, agarose gels should be run at no more than 5 V/cm

Distance is measured as the shortest path between the electrodes and is not merely the length of the gel itself

DIRECTION OF THE ELECTRIC FIELD

DNA molecules larger than 50–100 kb in length migrate through agarose gels at the same rate if the direction of the electric field remains constant However, if the direction of the electric field is altered periodically, the DNA molecules are forced to change course Because larger molecules of DNA take longer to realign themselves to the new direction of the field, pulsed-field gel electrophoresis can be used to fractionate populations of extremely large molecules of DNA (up to 10,000 kb). Pulsed-field gel electrophoresis is discussed in more detail beginning on page 6.50

BASE COMPOSITION AND TEMPERATURE

The electrophoretic behavior of DNA in agarose gels (in contrast to polyacrylamide gels [Allet et al 1973]) is not significantly affected by either the base composition of the DNA (Thomas and Davis 1975) or the temperature at which the gel is run Thus, in agarose gels, the relative electrophoretic mobilities of DNA fragments of different sizes do not change between 4°C and 30°C In general, agarose gels are run at room temperature However, gels containing less than 0 5% agarose and low-melting-temperature agarose gels are rather flimsy, and it is best to run them at 4°C, where they are stronger.

PRESENCE OF INTERCALATING DYES

Ethidium bromide, a fluorescent dye that is used to detect DNA in agarose and polyacrylamide gels (Sharp et al 1973), reduces the electrophoretic mobility of linear DNA by about 15% The dye intercalates between stacked base pairs, extending the length of linear and nicked circular DNA molecules and making them more rigid

Ethidium bromide is a carcinogen and should be handled with care. All solutions containing ethidium bromide should be decontaminated before disposal (see pages 6 16–6.17)

COMPOSITION OF THE ELECTROPHORESIS BUFFER

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions (e.g , if electrophoresis buffer is omitted from the gel by mistake), electrical conductance is minimal and DNA migrates very slowly, if at all. In buffers of high ionic strength (e.g., if 10 × electrophoresis buffer is used by mistake), electrical conductance is very efficient and significant amounts of heat are generated. In the worst case, the gel melts and the DNA denatures.

Several different buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50 mM (pH 7.5–7.8) (Table 6.2). Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature.

For historical reasons, TAE is the most commonly used buffer. However, its buffering capacity is rather low, and it tends to become exhausted during extended electrophoresis (the anode becomes alkaline, the cathode acidic). Replacement of the buffer or recirculation between the two reservoirs is therefore advisable when carrying out electrophoresis for long periods of time at high current. Both TPE and TBE are slightly more expensive than TAE, but they have significantly higher buffering capacity. Double-stranded linear DNA fragments migrate approximately 10% faster through TAE than through TBE or TPE, but the resolving powers of these systems are almost identical, with the exception that the resolution of supercoiled DNAs is better in TAE than in TBE.

The most commonly used buffer for electrophoresis of denatured single-stranded DNA is 50 mN NaOH, 1 mM EDTA (alkaline electrophoresis buffer; see Table 6.2). As discussed below, agarose cannot be melted in the presence of NaOH. Therefore, the agarose must be melted in water before the concentrated NaOH/EDTA solution is added.

TABLE 6.2 Commonly Used Electrophoresis Buffers

Buffer	Working solution	Concentrated stock solution (per liter)
Tris-acetate (TAE)	1× 0.04 M Tris-acetate 0.001 M EDTA	50× 242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)
Tris-phosphate (TPE)	1× 0.09 M Tris-phosphate 0.002 M EDTA	10× 108 g Tris base 15.5 ml 85% phosphoric acid (1.679 g/ml) 40 ml 0.5 M EDTA (pH 8.0)
Tris-borate[a] (TBE)	0.5× 0.045 M Tris-borate 0.001 M EDTA	5× 54 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8.0)
Alkaline[b]	1× 50 mN NaOH 1 mM EDTA	1× 5 ml 10 N NaOH 2 ml 0.5 M EDTA (pH 8.0)

[a] A precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid problems, store the 5× solution in glass bottles at room temperature and discard any batches that develop a precipitate.

TBE was originally used at a working strength of 1× (i.e., a 1:5 dilution of the concentrated stock) for agarose gel electrophoresis. However, a working solution of 0.5× provides more than enough buffering power, and almost all agarose gel electrophoresis is now carried out with a 1:10 dilution of the concentrated stock.

TBE is used at a working strength of 1× for polyacrylamide gel electrophoresis (see page 6.39), twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them is often considerable. 1× TBE is required to provide adequate buffering power.

[b] Alkaline electrophoresis buffer should be freshly made.

Apparatuses Used for Agarose Gel Electrophoresis

Because agarose gel electrophoresis is both forgiving and adaptable, many successful configurations and sizes of electrophoresis tanks have been designed during the last 15 years. The choice among these different designs is largely a matter of personal preference. The most commonly used configuration is the horizontal slab gel, which was invented by Walter Schaffner and is superior to the old-fashioned vertical gels in several respects.

- Low agarose concentrations can be used because the entire gel is supported from beneath
- Gels can be cast in a wide variety of sizes
- The gels are very simple to load, pour, and handle
- The apparatus is durable and inexpensive to construct or buy

Horizontal slab gels are usually poured on a glass plate or plastic tray that can be installed on a platform in the electrophoresis tank In a few designs, the gel is poured directly on the platform Electrophoresis is carried out with the gel submerged just beneath the surface of the buffer The resistance of the gel to the passage of electric current is almost the same as that of the buffer, and so a considerable fraction of the applied current passes along the length of the gel.

Among the factors to be considered when buying an electrophoresis tank are

- *It should be easy to examine the gel by ultraviolet light during the run* When the gel is poured on a glass plate or plastic tray, it can easily be removed, examined, and reinstalled at any stage. Most of the plastic trays currently supplied by commercial manufacturers are translucent to ultraviolet light, eliminating the need to transfer the gel from its support for examination When the gel is poured directly on a platform within the tank, however, it may be more difficult to remove the gel from the tank both during and at the end of the run
- *The apparatus should be supplied with a variety of combs to generate different numbers of wells of different sizes* It is an advantage if the gel can accommodate two rows of wells, with at least 16 wells in each row. This makes it possible to analyze many samples of DNA simultaneously
- *The apparatus should be fitted with a lid containing shielded electrical connections* It should be impossible to remove the lid while electric current is flowing through the system The tank should be designed to allow easy replacement of corroded or broken connections and the platinum electrodes.
- *The apparatus should be fitted with outlets to allow electrophoresis buffer to be removed easily and completely* In many older designs, the buffer was removed by inverting the tank over a sink. This is not acceptable when the buffer contains ethidium bromide.
- *The outlets should be designed to allow circulation of buffer between the anodic and cathodic chambers.* This is important when RNA is analyzed by electrophoresis.

PREPARATION AND EXAMINATION OF AGAROSE GELS

Preparation of an Agarose Gel

1. Seal the edges of a clean, dry, glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with autoclave tape so as to form a mold (Figure 6 2) Set the mold on a horizontal section of the bench (check with a level)

2. Prepare sufficient electrophoresis buffer (usually 1 × TAE or 0.5 × TBE; see Table 6 2, page 6 7) to fill the electrophoresis tank and to prepare the gel Add the correct amount of powdered agarose (see Table 6.1, page 6.5) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle with a loose-fitting cap The buffer should not occupy more than 50% of the volume of the flask or bottle

   It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel Small differences in ionic strength or pH create fronts in the gel that can greatly affect the mobility of DNA fragments

3. Loosely plug the neck of the Erlenmeyer flask with Kimwipes When using a glass bottle, make sure the cap is loose. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves

   Heat the slurry for the minimum time required to allow all of the grains of agarose to dissolve Wearing an oven mitt, gingerly swirl the bottle or flask from time to time to make sure that any grains sticking to the walls enter the solution *Take care—the agarose solution can become superheated and may boil violently if it has been heated for too long in the microwave oven* Undissolved agarose appears as small "lenses" floating in the solution Check that the volume of the solution has not been decreased by evaporation during boiling, replenish with water if necessary

4. Cool the solution to 60°C, and, if desired, add ethidium bromide (from a stock solution of 10 mg/ml in water) to a final concentration of 0 5 μg/ml and mix thoroughly

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 6 16–6 17

   Stock solutions of ethidium bromide should be stored in light-tight containers (e g , in a bottle completely wrapped in aluminum foil) at room temperature

   When preparing gels that contain high concentrations of agarose (2% or above), cool the solution quickly to 70°C and pour the gel immediately The higher the concentration of agarose, the quicker the gel hardens Problems caused by premature hardening of the gel can be avoided by using sieving grades of low-gelling-temperature agarose However, these grades of agarose are suitable only for analytical electrophoresis, since DNA fragments eluted from the gel are frequently contaminated with inhibitors that prevent further enzymatic manipulation

5. Using a pasteur pipette, seal the edges of the mold with a small quantity of the agarose solution. Allow the seal to set. Position the comb 0.5–1.0 mm above the plate so that a complete well is formed when the agarose is added If the comb is closer to the glass plate, there is a risk that the

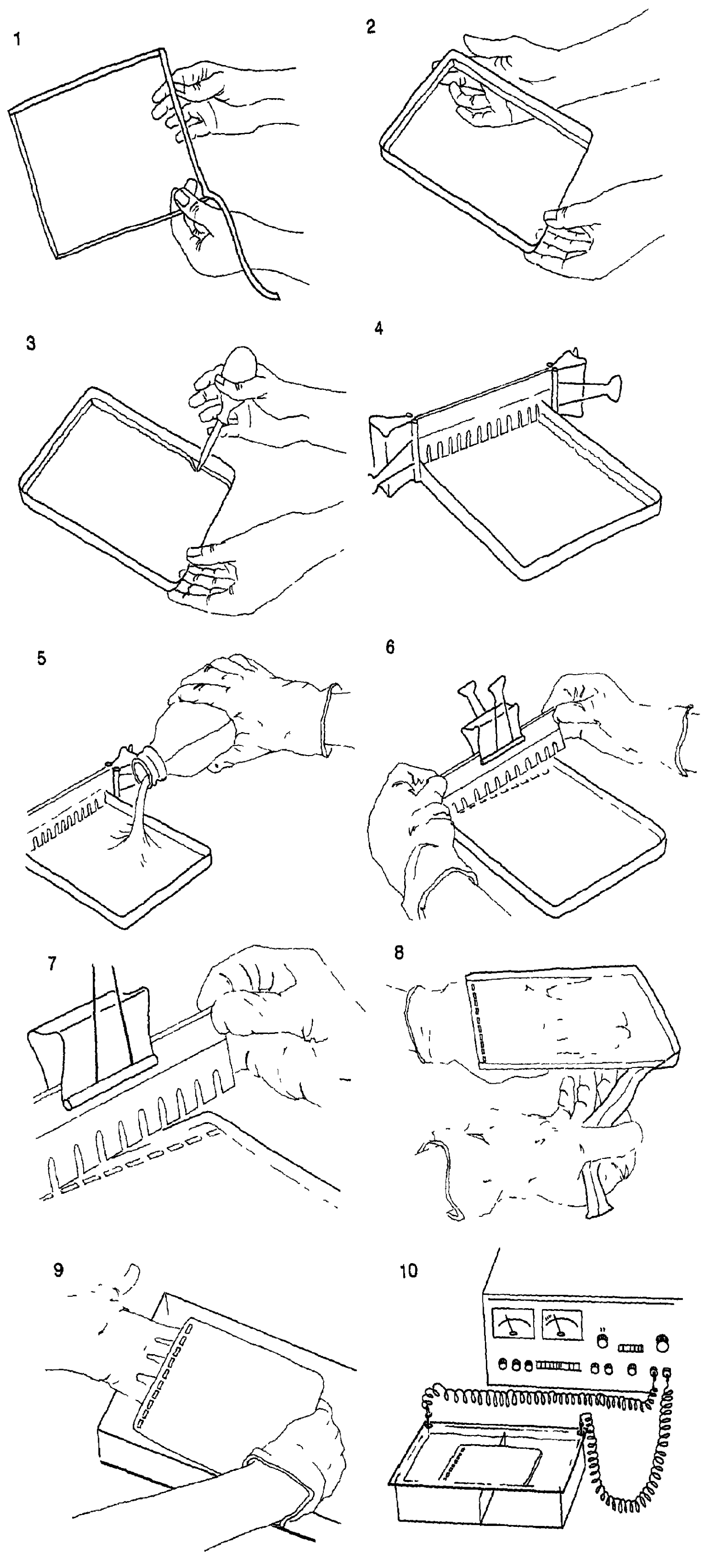


FIGURE 6.2
Pouring a horizontal agarose gel.

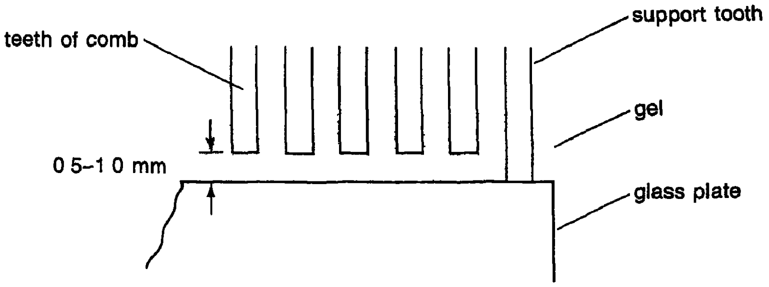


FIGURE 6.3
Diagram of a comb used to form slots in horizontal agarose gels

base of the well may tear when the comb is withdrawn, allowing the sample to leak between the gel and the glass plate.

Some combs are designed with two outer teeth slightly longer than the internal teeth When the comb is positioned above the plate, it is supported on the outer teeth, the inner teeth are automatically lifted clear of the glass plate (Figure 6 3) The disadvantage of this design is that the wells made by the outer teeth cannot be used during the subsequent electrophoresis

6. Pour the remainder of the warm agarose solution into the mold The gel should be between 3 mm and 5 mm thick. Check to see that there are no air bubbles under or between the teeth of the comb

   When preparing gels that contain low concentrations of agarose (<0 5%), first pour a supporting gel (1% agarose) without wells Allow this gel to harden at room temperature on the glass plate or plastic tray Then pour the lower-percentage gel directly on top of the supporting gel This reduces the chances that the lower-percentage gel will fracture during subsequent manipulations (photography, processing for Southern hybridization, etc) Make sure that both gels are made from the same batch of buffer and that ethidium bromide is added to both gels or to neither

7. After the gel is completely set (30–45 minutes at room temperature), carefully remove the comb and autoclave tape and mount the gel in the electrophoresis tank

   Gels cast with low-melting-temperature agarose and gels that contain less than 0 5% agarose should be chilled to 4°C and run in the cold room

8. Add just enough electrophoresis buffer to cover the gel to a depth of about 1 mm.

9. Mix the samples of DNA with the desired gel-loading buffer (Table 6 3) Slowly load the mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or, if you have a very steady hand, a pasteur pipette.

   Gel-loading buffers are usually made up as sixfold concentrated solutions (See Table 6 3 for the preparation of standard 6× buffers) Tenfold concentrated solutions can also be prepared if needed

   The maximum amount of DNA that can be applied to a slot depends on the number of fragments in the sample and their sizes The minimum amount of DNA that can be detected by photography of ethidium-bromide-stained gels is about 2 ng in a

0.5-cm-wide band (the usual width of a slot). If there is more than 500 ng of DNA in a band of this width, the slot will be overloaded, resulting in trailing and smearing—a problem that becomes more severe as the size of the DNA increases. When simple populations of DNA molecules (e.g., bacteriophage λ or plasmid DNAs) are to be analyzed, 100–500 ng of DNA should be loaded per 0.5-cm slot. When the sample consists of a very large number of DNA fragments of different sizes (e.g., restriction digests of mammalian DNA), however, it is possible to load 20–30 μg of DNA per slot without significant loss of resolution.

The maximum volume of solution that can be loaded is determined by the dimensions of the slot. (A typical slot [0.5 cm × 0.5 cm × 0.15 cm] will hold about 37.5 μl.) However, to reduce the possibility of contaminating neighboring samples, it is best to make the gel a little thicker or to concentrate the DNA by ethanol precipitation rather than to fill the slot completely.

For most purposes, it is not necessary to use a fresh pipette tip for every sample as long as the tip is thoroughly washed with buffer from the anodic chamber between samples. However, if the gel is to be analyzed by Southern hybridization or if bands of DNA are to be recovered from the gel, it is sensible to use a separate pipette tip for every sample.

Marker DNAs of known size (which can be purchased from commercial sources) should be loaded into slots on both the right and left sides of the gel. This makes it

TABLE 6.3 Gel-loading Buffers

Buffer type	6× Buffer	Storage temperature
I	0.25% bromophenol blue 0.25% xylene cyanol FF 40% (w/v) sucrose in water	4°C
II	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 400, Pharmacia) in water	room temp
III	0.25% bromophenol blue 0.25% xylene cyanol FF 30% glycerol in water	4°C
IV	0.25% bromophenol blue 40% (w/v) sucrose in water	4°C
V	*Alkaline loading buffer* 300 mN NaOH 6 mM EDTA 18% Ficoll (Type 400, Pharmacia) in water 0.15% bromocresol green 0.25% xylene cyanol FF	4°C

These gel-loading buffers serve three purposes. They increase the density of the sample, ensuring that the DNA drops evenly into the well; they add color to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels approximately 2.2-fold faster than xylene cyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5× TBE at approximately the same rate as linear double-stranded DNA 300 bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double-stranded DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5% to 1.4%.

Which type of loading dye to use is a matter of personal preference. However, bromocresol green should be used as a tracking dye in alkaline gels because it displays a more vivid color than bromophenol blue at alkaline pH.

easier to determine the sizes of unknown DNAs if any systematic distortion of the gel should occur during electrophoresis

When measuring the sizes of unknown DNAs, it is important that all samples be applied to the gel in the same buffer The high concentrations of salt in certain restriction enzyme buffers (e g , *Bam*HI or *Eco*RI) retard the migration of DNA and distort the electrophoresis of DNA in the adjacent wells

10. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a voltage of 1–5 V/cm (measured as the distance between the electrodes). If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and, within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance through the gel

During electrophoresis, the ethidium bromide migrates toward the cathode (in the direction opposite to that of the DNA) Extended electrophoresis can remove much of the ethidium bromide from the gel, making detection of small fragments difficult If this occurs, restain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0 5 μg/ml) as described on page 6 15

The presence of ethidium bromide allows the gel to be examined by ultraviolet illumination at any stage during electrophoresis However, some people feel that sharper bands of DNA are obtained when the gel is run in the absence of the dye In this case, after electrophoresis is completed, stain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0 5 μg/ml) as described on page 6 15

11. Turn off the electric current and remove the leads and lid from the gel tank If ethidium bromide was present in the gel and electrophoresis buffer, examine the gel by ultraviolet light and photograph the gel as described on page 6 19. Otherwise, stain the gel with ethidium bromide as described on page 6.15 and then photograph.

Solutions containing ethidium bromide should be decontaminated by one of the methods described on pages 6 16–6 17 before they are discarded

Minigels

During the last 5 years, methods have been developed for analyzing small quantities of DNA very rapidly by using miniature agarose gels Several types of miniature electrophoresis tanks are manufactured commercially. Because of the rather delicate milling involved in shaping the tiny combs and other parts, these tanks are quite expensive However, inexpensive versions can be constructed easily in the laboratory

A convenient electrophoresis chamber for submerged minigels is a small (2 1/2 inches × 6 1/2 inches) polystyrene snap-lock box Four inches of platinum wire are required for each of the two electrodes, which are glued in place with silicone rubber cement. A platform composed of four 5-cm × 7.5-cm lantern slides glued together in a stack is cemented in the center of the box (Figure 6.4)

The gels themselves, consisting of 10–12 ml of melted agarose solution (0.5–2 0%) containing ethidium bromide (0 5 μg/ml), are poured on 5-cm × 7 5-cm lantern slides using a disposable 10-ml pipette The solution flows better when the pipette tip is broken off. Each gel can accommodate eight slots, 2.5 mm long × 1 mm wide, which are formed by using a miniature Lucite comb of standard design. Several gels may be prepared simultaneously by mounting a long gel comb across slides placed side by side on a sheet of Parafilm

Ethidium bromide is almost always included to reduce the time required between ending the run and seeing the result By having a large number of lantern slides on hand, many gels can be poured at once After hardening, the gels are wrapped in Saran Wrap, labeled (with the date and the concentration of the agarose), and stored in a drawer in the dark at room temperature Either the whole minigel may be used at once or as many lanes as are

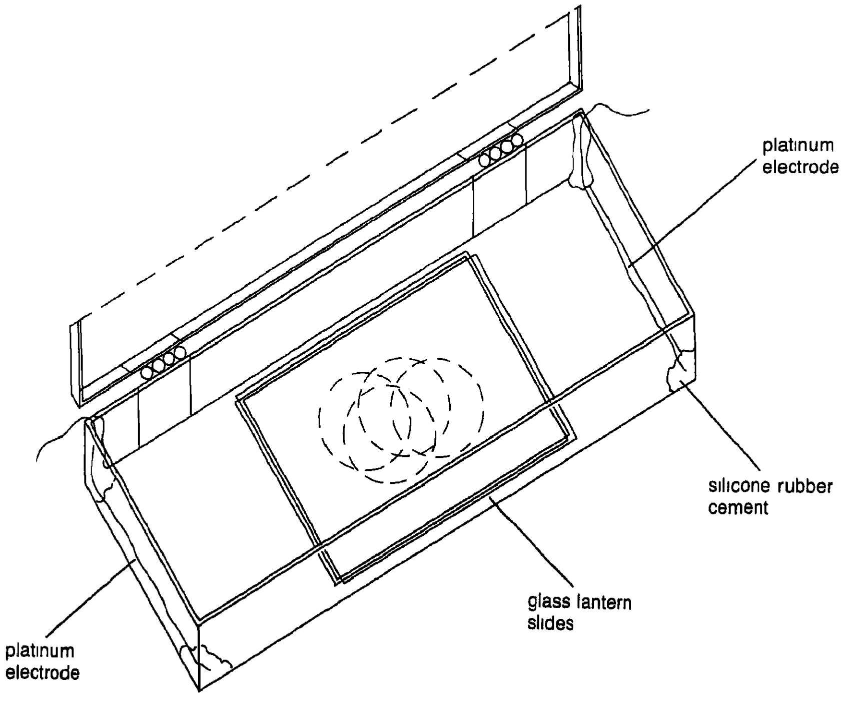


FIGURE 6.4
A minigel apparatus that can be constructed inexpensively in the laboratory.

required may simply be cut from the gel with a razor blade and carefully transferred to the electrophoresis apparatus

Each gel slot holds 3–12 μl of fluid, depending on the thickness of the gel and the width of the teeth of the comb Usually, 10–100 ng of DNA, in the gel-loading buffer of choice, are applied to a slot The gel is run for 30–60 minutes at high voltage (5–20 V/cm) until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance. The gel is then photographed as described on page 6.19.

Minigels are particularly useful when a rapid answer is required before the next step in a cloning protocol can be undertaken Because the wells are smaller and the gels are thinner, less DNA than normal is required for visualization. Also, because the gels can be prepared in advance and run rapidly and because they require smaller amounts of reagents, there are considerable savings in both time and money. However, minigels are best suited for the analysis of small DNA fragments (<3 kb). Larger fragments are resolved poorly because of the high voltages that are generally used and the comparatively short length of the gel

Staining DNA in Agarose Gels

The most convenient method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide (Sharp et al 1973) This substance contains a planar group that intercalates between the stacked bases of DNA. The fixed position of this group and its close proximity to the bases cause dye bound to DNA to display an increased fluorescent yield compared to that of dye in free solution Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye, radiation at 302 nm and 366 nm is absorbed by the bound dye itself In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum Because the fluorescent yield of ethidium bromide DNA complexes is much greater than that of unbound dye, small amounts of DNA can be detected in the presence of free ethidium bromide in the gel

Ethidium bromide can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA) However, the affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor

Ethidium bromide is usually prepared as a stock solution of 10 mg/ml in water, which is stored at room temperature in dark bottles or bottles wrapped in aluminum foil The dye is usually incorporated into the gel and the electrophoresis buffer at a concentration of 0 5 μg/ml. Although the electrophoretic mobility of linear double-stranded DNA is reduced by approximately 15% in the presence of the dye, the ability to examine the gel directly under ultraviolet illumination during or at the end of the run is often a great advantage However, the gel may also be run in the absence of ethidium bromide and stained after electrophoresis is complete In this case, the gel is immersed in electrophoresis buffer or water containing ethidium bromide (0 5 μg/ml) for 30–45 minutes at room temperature. Destaining is not usually required However, detection of very small amounts (<10 ng) of DNA is made easier if the background fluorescence caused by unbound ethidium bromide is reduced by soaking the stained gel in water or 1 mM $MgSO_4$ for 20 minutes at room temperature.

Decontamination of Ethidium Bromide Solutions

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described below

DECONTAMINATION OF CONCENTRATED SOLUTIONS OF ETHIDIUM BROMIDE (i.e., solutions containing >0.5 mg/ml)

Method 1

This method (Lunn and Sansone 1987) reduces the mutagenic activity of ethidium bromide in the *Salmonella*/microsome assay by approximately 200-fold.

1. Add sufficient water to reduce the concentration of ethidium bromide to <0.5 mg/ml

2. To the resulting solution, add 0.2 volume of fresh 5% hypophosphorous acid and 0.12 volume of fresh 0.5 M sodium nitrite Mix carefully
   Important: Check that the pH of the solution is <3.0

   Hypophosphorous acid is usually supplied as a 50% solution, which is corrosive and should be handled with care It should be freshly diluted immediately before use

   Sodium nitrite solution (0.5 M) should be freshly prepared by dissolving 34.5 g of sodium nitrite in water to a final volume of 500 ml

3. After incubation for 24 hours at room temperature, add a large excess of 1 M sodium bicarbonate The solution may now be discarded

Method 2

This method (Quillardet and Hofnung 1988) reduces the mutagenic activity of ethidium bromide in the *Salmonella*/microsome assay by approximately 3000-fold. However, there are reports (Lunn and Sansone 1987) of mutagenic activity in occasional batches of "blanks" treated with the decontaminating solutions.

1. Add sufficient water to reduce the concentration of ethidium bromide to <0.5 mg/ml.

2. Add 1 volume of 0.5 M $KMnO_4$. Mix carefully, and then add 1 volume of 2.5 N HCl. Mix carefully, and allow the solution to stand at room temperature for several hours

3. Add 1 volume of 2.5 N NaOH. Mix carefully, and then discard the solution.

DECONTAMINATION OF DILUTE SOLUTIONS OF ETHIDIUM BROMIDE
(e.g., electrophoresis buffer containing 0.5 μg/ml ethidium bromide)

Method 1

The following method is from Lunn and Sansone (1987).

1. Add 2.9 g of Amberlite XAD-16 for each 100 ml of solution. Amberlite XAD-16, a nonionic, polymeric absorbent, is available from Rohm and Haas.
2. Store the solution for 12 hours at room temperature, shaking it intermittently
3. Filter the solution through a Whatman No. 1 filter, and discard the filtrate
4. Seal the filter and Amberlite resin in a plastic bag, and dispose of the bag in the hazardous waste.

Method 2

The following method is from Bensaude (1988)

1. Add 100 mg of powdered activated charcoal for each 100 ml of solution
2. Store the solution for 1 hour at room temperature, shaking it intermittently
3. Filter the solution through a Whatman No. 1 filter, and discard the filtrate
4. Seal the filter and activated charcoal in a plastic bag, and dispose of the bag in the hazardous waste.

Notes

i. Treatment of dilute solutions of ethidium bromide with hypochlorite (bleach) is not recommended as a method of decontamination Such treatment reduces the mutagenic activity of ethidium bromide in the *Salmonella*/microsome assay by about 1000-fold, but it converts the dye into a compound that is mutagenic in the absence of microsomes (Quillardet and Hofnung 1988).

ii Ethidium bromide decomposes at 262°C and is unlikely to be hazardous after incineration under standard conditions.

iii. Slurries of Amberlite XAD-16 or activated charcoal can be used to decontaminate surfaces that become contaminated by ethidium bromide.

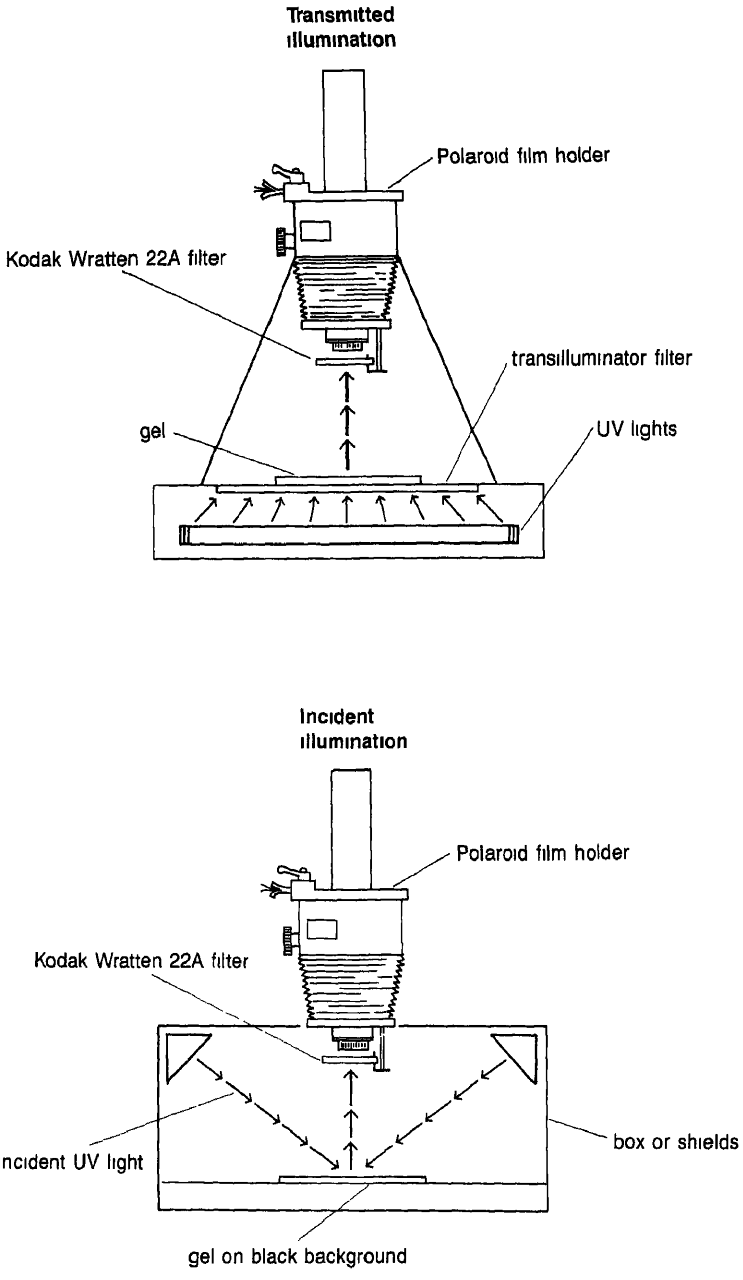


FIGURE 6.5
Photography of gels by ultraviolet illumination The top diagram shows the arrangement of the ultraviolet light source, the gel, and the camera that is used for photography by transmitted light The bottom diagram shows the arrangement that is used for photography by incident light

Photography

Photographs of gels may be made using transmitted or incident ultraviolet light (Figure 6.5). Most commercially available ultraviolet light sources emit ultraviolet light at 302 nm. The fluorescent yield of ethidium bromide:DNA complexes is considerably greater at this wavelength than at 366 nm and slightly less than at short-wavelength (254 nm) light. However, the amount of nicking of the DNA is much less at 302 nm than at 254 nm.

The most sensitive film is Polaroid Type 57 or 667 (ASA 3000). With an efficient ultraviolet light source ($>2500\ \mu W/cm^2$), a Wratten 22A filter, and a good lens ($f = 135$ mm), an exposure of a few seconds is sufficient to obtain images of bands containing as little as 10 ng of DNA. With a long exposure time and a strong ultraviolet light source, the fluorescence emitted by as little as 1 ng of DNA can be recorded on film. For detection of extremely faint bands, a lens with a shorter focal length ($f = 75$ mm) should be used in combination with conventional wet-process film (e.g., Eastman Kodak No. 4155). This allows the lens to be moved closer to the gel, concentrates the image on a smaller area of film, and allows for flexibility in developing and printing the image.

Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

Alkaline Agarose Gels

Alkaline agarose gels (McDonell et al. 1977) are used to analyze single-stranded DNA. In particular, they are used for the following purposes:

- To analyze the size of the cDNA strand in nuclease-S1-resistant DNA:RNA hybrids (see Favaloro et al. 1980)
- To check the size of the first and second strands of cDNA synthesized by reverse transcriptase
- To check for nicking activity in enzyme preparations used for molecular cloning
- To calibrate the reagents used in nick translation of DNA

Because the addition of NaOH to a hot agarose solution causes hydrolysis of the polysaccharide, the agarose is melted in a neutral, unbuffered solution (usually water) and equilibrated in alkaline electrophoresis buffer before the gel is poured Formamide and urea are not used as denaturants because they cause agarose gels to become rubbery

1. Add the correct amount of powdered agarose (see Table 6.1, page 6 5) to a measured quantity of water in an Erlenmeyer flask or a glass bottle with a loose-fitting cap

2. Loosely plug the neck of the Erlenmeyer flask with Kimwipes When using a glass bottle, make sure the cap is loose Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves

   Heat the slurry for the minimum time required to allow all of the grains of agarose to dissolve Wearing an oven mitt, gingerly swirl the bottle or flask from time to time to make sure that any grains sticking to the walls enter the solution *Take care—the agarose solution can become superheated and may boil violently if it has been heated for too long in the microwave oven* Undissolved agarose appears as small "lenses" floating in the solution Check that the volume of the solution has not been decreased by evaporation during boiling, replenish with water if necessary

3. Cool the solution to 60°C Add NaOH to 50 mM and EDTA (pH 8.0) to 1 mM, and then pour the gel as described on page 6.11 After the gel is completely set, mount it in the electrophoresis tank and add 1 × alkaline electrophoresis buffer (see Table 6 2, page 6.7) until the gel is just covered.

   Ethidium bromide is always omitted from alkaline agarose gels because it does not bind to DNA at high pH

4. Precipitate the samples of DNA to be analyzed with 2 volumes of ethanol at 0°C, and dissolve the precipitate in 10–20 μl of 50 mN NaOH, 1 mM EDTA. Add 0 2 volume of 6 × alkaline loading buffer (see Table 6 3, page 6.12). Alternatively, if the volumes of the original DNA samples are small (<15 μl), add EDTA (pH 8.0) to a final concentration of 10 mM followed by 0 2 volume of 6 × alkaline loading buffer

5. Load the DNA samples dissolved in 6 × alkaline loading buffer into the wells of the gel as described on page 6.11. Carry out electrophoresis until the dye has migrated approximately two thirds of the length of the gel.

Alkaline gels draw more current than neutral gels at comparable voltages, and they heat up during the run Alkaline agarose electrophoresis should therefore be carried out at voltages of < 0 25 V/cm

Because bromocresol green diffuses rapidly out of alkaline gels into the electrophoresis buffer, a glass plate should be placed directly on top of the gel *after* the dye has migrated out of the loading slot The alkaline electrophoresis buffer also causes the gel to detach from the baseplate and to float free in the buffer The weight of the upper plate helps to hold the gel in place

6. In many cases, DNA analyzed by alkaline agarose gel electrophoresis is labeled with ^{32}P, which can be detected by autoradiography. At the end of the run, remove the gel from the tank and soak it in 7% trichloroacetic acid (TCA) for 30 minutes at room temperature. After soaking, mount the gel on a glass plate and dry it for several hours under layers of paper towels weighted with another glass plate, or dry it under vacuum on a gel dryer Heat should not be used because of the possibility of melting the gel. Cover the dried gel with Saran Wrap and attach adhesive dot labels marked with radioactive ink to align the film with the gel Cover the labels with Scotch Tape This prevents radioactive contamination of the film holder or intensifying screen Autoradiograph the gel at room temperature or at −70°C with an intensifying screen (see Appendix E)

If a fragment is to be recovered from an alkaline agarose gel, do not soak the gel in 7% TCA Instead, either autoradiograph the wet gel (using adhesive labels marked with radioactive ink to align the film and the gel) or neutralize the gel by soaking it in 1 M Tris Cl (pH 7 6), 1 5 M NaCl for 45 minutes followed by staining with 0 5 μg/ml ethidium bromide in 1 × TAE The band of interest can then be sliced from the gel and subsequently eluted by one of the procedures described on pages 6 22–6 31

Drying the gel greatly improves the sharpness of the autoradiographic image and slightly increases the sensitivity because of the elimination of quenching However, if there is sufficient radiolabel in the DNA and the sharpness of the bands is not a major concern, the gel can be autoradiographed without drying After completion of electrophoresis, place the gel on a glass plate Slide the gel and plate carefully into a thin plastic bag (e g , Sears Seal-A-Meal), squeeze the air from the bag, and seal the top of the bag in a heat sealer Alternatively, wrap the gel and backing plate in Saran Wrap This procedure is less satisfactory because radioactive fluid tends to ooze out of the Saran Wrap during autoradiography Apply adhesive labels marked with radioactive ink to the outside of the plastic bag or Saran Wrap In a darkroom, tape the sealed gel to a piece of X-ray film (Kodak XAR-2 or equivalent) cut to the same size as the glass plate *Do not use a metal film cassette* (which may break the glass plate and crush the gel) Instead, wrap the gel and film in light-tight aluminum foil Expose the film for an appropriate period of time (see Appendix E)

Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black ink We find it convenient to make the ink in three grades very hot (> 2000 cps on a hand-held minimonitor), hot (> 500 cps on a hand-held minimonitor), and cool (> 50 cps on a hand-held minimonitor) Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels Attach radioactive-warning tape to the pen and store it in an appropriate place

If the DNA is unlabeled, the gel should be soaked for 45 minutes in neutralizing solution (1 M Tris · Cl [pH 7 6], 1 5 M NaCl) The DNA can then be transferred to a nitrocellulose filter as described in Chapter 9, pages 9.34–9.41, and detected by hybridization to an appropriate ^{32}P-labeled probe. Alternatively, the DNA can be transferred directly from the alkaline agarose gel to a nylon membrane (see Chapter 9, pages 9 45–9.46).

RECOVERY AND PURIFICATION OF DNA FRACTIONATED ON AGAROSE GELS

Although many methods have been developed over the years to recover DNA from gels, none has proved entirely satisfactory. Among the problems are·

- *The presence of inhibitors of enzymatic reactions.* In the past, most grades of agarose were contaminated with poorly characterized polysaccharides, which are extracted from the gel together with the DNA These substances are potent inhibitors of many of the enzymes commonly used in subsequent cloning steps. Although improvements in the quality of agarose over the past few years have reduced this problem considerably, there are still occasions when DNA is recovered in an unreactive form.
- *Inefficient recovery of large fragments of DNA.* The efficiency with which DNA is recovered from agarose gels is a function of its molecular weight Although most methods give reasonable yields of DNA fragments that are less than 5 kb in length (> 50%), none of them is entirely satisfactory for the recovery of larger fragments. As the size of the DNA fragments increases, the yield progressively decreases, especially when the DNA is purified by methods that involve binding to a solid matrix such as DEAE-Sephacel or DEAE-cellulose membrane Larger fragments bind more tenaciously to such supports and are more difficult to elute
- *Inefficient recovery of small amounts of DNA* The smaller the amount of DNA applied to the gel, the lower the yield of purified fragments. In some methods, the loss of material is so great that it is not worthwhile attempting to recover bands that contain less than 500 ng of DNA
- *Inability to recover a number of different fragments simultaneously.* Several of the techniques are labor-intensive and consist of many individual manipulations The number of fragments that can be processed at any one time is therefore limited.

Of the many techniques available to recover DNA from gels, we have chosen to present the following three, which are the most reliable in our hands.

- Electrophoresis onto DEAE-cellulose membrane
- Electroelution into dialysis bags
- Low-melting-temperature agarose gels

Although these three techniques collectively meet all of the desired criteria, no single technique meets them all. Electrophoresis onto DEAE-cellulose membrane is a relatively simple technique that can be performed simultaneously on many samples and consistently gives high yields of fragments between 500 bp and 5 kb in length The DNA recovered from the membrane is of high purity and can be used for the most demanding tasks (e.g., the production of lines of transgenic mice) Electroelution into dialysis bags is by far the most inconvenient of these techniques; however, it is also the most effective for the recovery of large fragments of DNA (> 5 kb in length). The recovery of DNA from gels cast with low-melting-temperature agarose is less reproducible than the other two methods, but this method has the advantage that certain enzyme reactions (e g , digestion with restriction enzymes and ligation) can be carried out directly in the melted gel

Electrophoresis onto DEAE-cellulose Membrane

In this method, fragments of DNA are separated by electrophoresis through an agarose gel of the appropriate concentration A slit is then cut in the gel immediately ahead of the DNA fragment(s) of interest and a sliver of DEAE-cellulose membrane (e g., Schleicher and Schuell NA-45) is inserted into the slit. Electrophoresis is continued until all of the DNA in the band has been collected onto the membrane. The membrane is then removed from the slit and washed free of contaminants in a buffer of low ionic strength. Finally, the DNA is eluted from the membrane in a buffer of high ionic strength.

The following protocol is based on the procedure of Girvitz et al. (1980), who used a dialysis membrane to trap the DNA The original procedure was later modified by Dretzen et al. (1981), who replaced the dialysis membrane with DEAE paper However, the recovery of DNA is much more efficient when a DEAE-cellulose membrane is used. The procedure is very simple, can be applied to many fragments simultaneously, and yields DNA that is extremely pure. DNA prepared by this method can therefore be used for such demanding tasks as microinjection of fertilized mouse eggs. Although the method is very efficient for DNA molecules that are less than 5 kb in length, larger DNA molecules are eluted from the membrane with progessively decreasing efficiency The method is therefore unsuitable for DNA fragments larger than 15 kb in size. Electrophoresis onto DEAE-cellulose membrane also cannot be used to isolate single-stranded DNAs, which are difficult to elute from the membrane.

1. Digest an amount of DNA that will yield at least 100 ng of the fragment(s) of interest Separate the fragments by electrophoresis through an agarose gel of the appropriate concentration that contains 0 5 μg/ml ethidium bromide Locate the band of interest with a hand-held, long-wavelength ultraviolet lamp This minimizes radiation damage to the DNA.

   Cautions: Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

   Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 6.16–6 17.

2. Using a sharp scalpel or razor blade, make an incision in the gel directly in front of the leading edge of the band of interest and about 2 mm wider than the band on each side.

   If DNA is to be eluted from an entire lane (e g , a restriction digest of mammalian genomic DNA), make the incision in the gel parallel to the lane of interest and place a single long piece of DEAE-cellulose membrane (prepared as in step 3) into the incision Reorient the gel so that the DNA can be transferred electrophoretically from the gel to the membrane After electrophoresis, remove the membrane and cut it into segments Elute DNA of the desired size from the appropriate segment(s) of the membrane (see below)

3. Wearing gloves, cut a piece of DEAE-cellulose membrane (e.g , Schleicher and Schuell NA-45) that is the same width as the incision and slightly deeper (1 mm) than the gel. Soak the membrane for 5 minutes in 10 mM EDTA (pH 8 0). Replace the EDTA with 0 5 N NaOH, and soak the membrane for a further 5 minutes. Wash the membrane six times in sterile water The strips may be stored at 4°C in sterile water for several weeks after they have been activated

   Do not use larger pieces of membrane than necessary, since this reduces the efficiency with which DNA can be eluted

4. Using blunt-ended forceps (e g , Millipore forceps), hold apart the walls of the incision and insert the membrane into the slit

5. Remove the forceps and close the incision, being careful not to trap air bubbles

6. Resume electrophoresis (5 V/cm) until the band of DNA has migrated onto the membrane Follow the progress of the electrophoresis with a hand-held, long-wavelength ultraviolet lamp

   Electrophoresis should be continued for the minimum time necessary to transfer the DNA from the gel to the membrane Extended electrophoresis can result in cross contamination with other DNA fragments (see below) or unnecessary accumulation of contaminants from the agarose To monitor the progress of the electrophoresis in a convenient and inexpensive way, cover the gel with a cardboard box that contains an armhole on one side for a hand-held ultraviolet lamp and a small hole on the top for visualization

   To minimize the chances of contamination with unwanted species of DNA, either
   - cut out a segment of gel containing the band of interest and transfer it to a hole of the appropriate size cut in another region of the gel far from any other species of DNA, or
   - insert a second piece of membrane above the band of interest to trap unwanted species of DNA

7. When all of the DNA has left the gel and is trapped on the membrane, turn off the electric current Using blunt-ended forceps, remove the membrane from the gel and rinse it in 5–10 ml of low-salt wash buffer at room temperature This removes pieces of agarose from the membrane

   Do not allow the membrane to dry, otherwise, the DNA becomes irreversibly bound

Low-salt wash buffer

50 mM Tris · Cl (pH 8.0)
0.15 M NaCl
10 mM EDTA (pH 8.0)

8. Transfer the membrane to a microfuge tube. Add enough high-salt elution buffer to cover the membrane completely. The membrane should be crushed or folded gently, but not tightly packed. Cap the tube and incubate for 30 minutes at 65°C. Check the tube from time to time to ensure that the membrane does not expand above the level of the buffer.

   High-salt elution buffer

   50 mM Tris · Cl (pH 8.0)
   1 M NaCl
   10 mM EDTA (pH 8.0)

9. Transfer the fluid to a fresh microfuge tube. Add a second aliquot of high-salt elution buffer to the membrane, and incubate for a further 15 minutes at 65°C. Combine the two aliquots of high-salt buffer. Check that the membrane no longer contains a visible smear of ethidium-bromide-stained DNA. Discard the used membrane.

10. Extract the eluate once with phenol.chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 0.2 volume of 10 M ammonium acetate and 2 volumes of ethanol at 4°C. Store the mixture for 10 minutes at room temperature, and recover the DNA by centrifugation at 12,000*g* for 20 minutes at room temperature in a microfuge. Carefully rinse the pellet with 70% ethanol, and then redissolve the DNA in 3–5 μl of TE (pH 7.6).

    The addition of 10 μg of carrier RNA before precipitation may improve the recovery of small amounts of DNA. However, before adding the RNA, make sure that the presence of RNA will not compromise any subsequent reactions with the DNA.

    Carrier RNA can be prepared by dissolving commercially available yeast tRNA at a concentration of 10 mg/ml in sterile TE (pH 7.6) containing 0.1 M NaCl. The solution is then extracted twice with phenol (equilibrated in Tris · Cl [pH 7.6] [see Appendix B]) and twice with chloroform, and the RNA is precipitated with 2.5 volumes of ethanol at room temperature. The RNA is recovered by centrifugation at 5000*g* for 15 minutes at 4°C, redissolved at a concentration of 10 mg/ml in sterile TE (pH 7.6), divided into small aliquots, and stored at −20°C.

    When exceptionally pure DNA is required (e.g., for microinjection of mouse eggs), reprecipitate the DNA with ethanol as follows. Resuspend the precipitate in 200 μl of TE (pH 7.6), add 25 μl of 3 M sodium acetate (pH 5.2), and reprecipitate the DNA with 2 volumes of ethanol at 4°C. Recover the DNA by centrifugation (12,000*g* for 15 minutes at 4°C should be adequate in most cases). Carefully rinse the pellet with 70% ethanol, and then redissolve the DNA in 3–5 μl of TE (pH 7.6).

11. Check the amount and quality of the DNA by gel electrophoresis.

    a. Mix a small aliquot (estimated to contain 50 ng) of the final preparation of the fragment with 10 μl of TE (pH 7.6), and add 2 μl of the desired gel-loading buffer (see Table 6.3, page 6.12).

    b. Load and run an agarose gel of the appropriate concentration, using as markers appropriate restriction digests of known quantities of the original DNA. The isolated fragment should comigrate with the correct fragment in the restriction digest

c. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

Electroelution into Dialysis Bags

Although this technique allows the recovery of a wide size range of DNAs, it is very inconvenient. It should therefore be used only when it is necessary to isolate large fragments of DNA (> 5 kb in length) that are inefficiently recovered by other techniques.

1. Digest an amount of DNA that will yield approximately 1 μg of the fragment(s) of interest. Separate the fragments by electrophoresis through an agarose gel of the appropriate concentration that contains 0 5 μg/ml ethidium bromide Locate the band of interest with a hand-held, long-wavelength ultraviolet lamp This minimizes radiation damage to the DNA.

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

2. Using a sharp scalpel or razor blade, cut out a slice of agarose containing the band of interest, and place it on a troughlike spatula that has been wetted with 1 × TAE electrophoresis buffer (see Table 6.2, page 6 7).

   Cut the smallest slice of agarose possible in order to reduce the amount of contamination of DNA with inhibitors

3. After cutting out the band, photograph the gel as described on page 6.19 so that there is a record of which band was eluted

4. Seal one end of a piece of dialysis tubing with a dialysis clip (see Appendix E for preparation of dialysis tubing) Fill the dialysis bag to overflowing with 1 × TAE Holding the neck of the bag, use the spatula to transfer the slice of agarose into the buffer-filled bag.

5. Allow the gel slice to sink to the bottom of the bag Remove most of the buffer, leaving just enough to keep the gel slice in constant contact with the buffer. Then clip the bag just above the gel slice; avoid trapping air bubbles (Figure 6.6)

6. Immerse the bag in a shallow layer of 1 × TAE in an electrophoresis tank Pass electric current through the bag (usually 4–5 V/cm for 2–3 hours) During this time, the DNA is electroeluted out of the gel and onto the inner wall of the bag The process can be conveniently monitored with a hand-held, long-wavelength ultraviolet lamp if sufficient DNA is available

7. Reverse the polarity of the current for 1 minute to release the DNA from the wall of the bag. Recover the bag from the electrophoresis chamber, and gently massage the side of the bag where the DNA accumulated to remove the DNA from the wall. The process can be followed with a hand-held, long-wavelength ultraviolet lamp if sufficient DNA is available.

8. Open the bag, and carefully transfer all of the buffer surrounding the gel slice to a fresh disposable plastic tube. Using a pasteur pipette, wash out the bag with a small quantity of 1 × TAE, and add the wash to the tube.

9. Stain the gel slice by immersing it in 1 × TAE containing ethidium bromide (0 5 μg/ml) for 30–45 minutes at room temperature Examine the stained slice by ultraviolet illumination to confirm that all the DNA has eluted

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described on pages 6.16–6 17

10. Purify the DNA either by passage through DEAE-Sephacel (see pages 6 32–6.33) or by extraction with organic solvents (see pages 6 34–6.35).

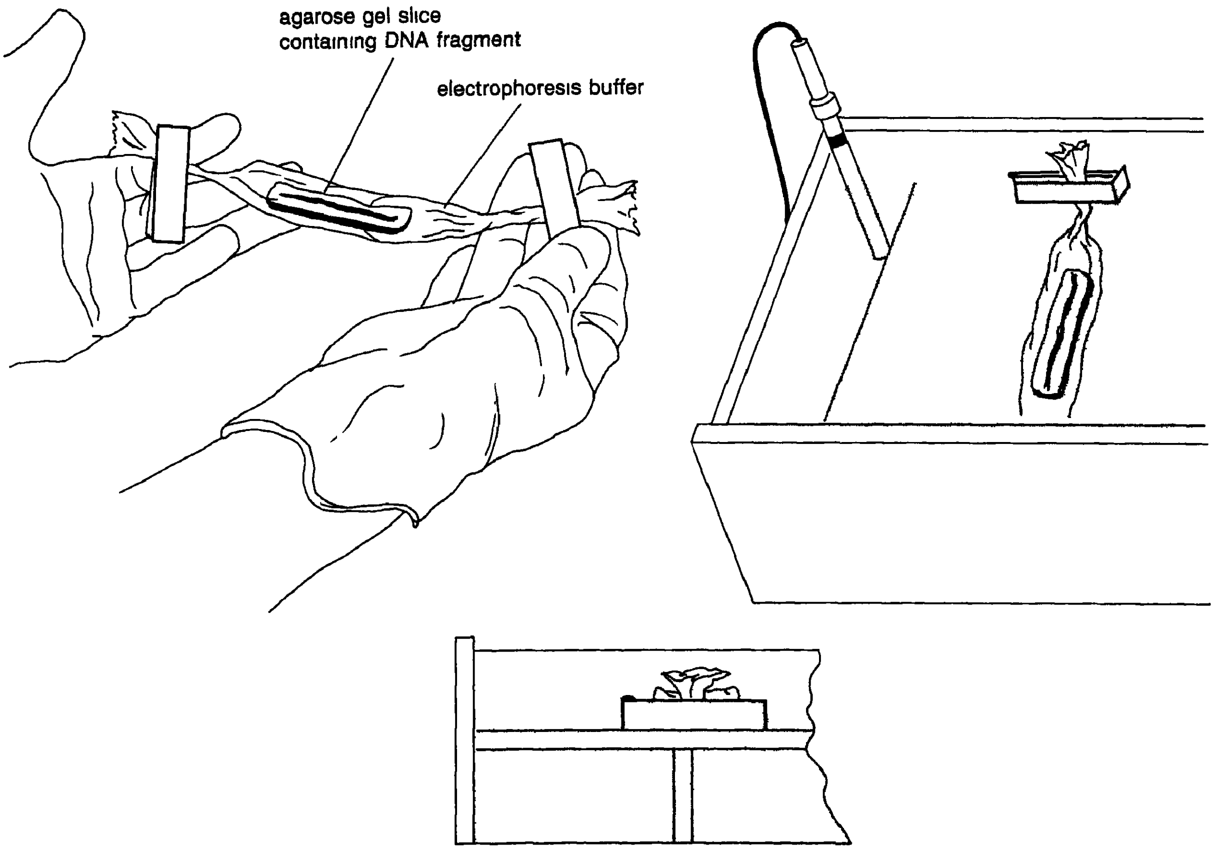

agarose gel slice containing DNA fragment
electrophoresis buffer

FIGURE 6.6
This method of dialysis was originally described by McDonell et al (1977)

Recovery of DNA from Low-melting-temperature Agarose Gels

A number of types of agarose are available in which hydroxyethyl groups have been introduced into the polysaccharide chain. This substitution causes the agarose to gel at approximately 30°C and to melt at approximately 65°C—well below the melting temperature of most double-stranded DNAs These properties have been exploited to develop a simple technique for the recovery of DNA from gels (Wieslander 1979; Parker and Seed 1980)

1. Pour a gel containing the appropriate concentration of low-melting-temperature agarose as described on pages 6.9–6 11 When the gel has cooled to room temperature, transfer it and its supporting glass plate to a horizontal surface in a cold room to ensure that the gel sets completely

2. Mix the samples of DNA with gel-loading buffer (see Table 6 3, page 6 12), load onto the gel, and carry out electrophoresis at 4°C to ensure that the gel does not melt during the run

   DNA of a given size runs slightly faster through gels cast with low-melting-temperature agarose than through conventional agarose gels

3. Locate the band of interest using a hand-held, long-wavelength ultraviolet lamp. This minimizes radiation damage to the DNA

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

4. Using a sharp scalpel or razor blade, cut out a slice of agarose containing the band of interest and transfer it to a clean, disposable plastic tube

   Cut the smallest slice of agarose possible in order to reduce the amount of contamination of DNA with inhibitors

5. After cutting out the band, photograph the gel as described on page 6.19 so that there is a record of which band was eluted

6. Add about 5 volumes of 20 mM Tris Cl (pH 8 0), 1 mM EDTA (pH 8 0) to the slice of agarose Close the top of the tube and incubate for 5 minutes at 65°C to melt the gel

7. Cool the solution to room temperature, and then add an equal volume of phenol, equilibrated to pH 8 0 with 0 1 M Tris · Cl. Vortex the mixture for 20 seconds, and then recover the aqueous phase by centrifugation at 4000g for 10 minutes at 20°C. The white substance at the interface is powdered agarose. Re-extract the aqueous phase once with phenol:chloroform and once with chloroform.

8. Transfer the aqueous phase to a polystyrene centrifuge tube, and add 0.2 volume of 10 M ammonium acetate and 2 volumes of ethanol at 4°C. Store the mixture for 10 minutes at room temperature, and then recover the

DNA by centrifugation (e.g , at 5000 rpm for 20 minutes at 4°C in a Sorvall RT6000 centrifuge) Usually, the DNA is now pure enough to serve as a substrate for restriction endonucleases, ligases, etc. However, if necessary, the DNA can be further purified by passage through DEAE-Sephacel (see pages 6 32–6 33)

Notes

i Because low-melting-temperature agarose remains fluid at 37°C, enzymatic manipulations (such as ligation, synthesis of radioactive probes, and digestion with restriction enzymes) can be carried out by adding portions of the melted gel slice directly to the reaction mixture (Parker and Seed 1980, Struhl 1985) (see, e g , Chapter 1, pages 1 72–1.73)

ii Recently an alternative method has been described to recover DNA from gels cast with low-melting-temperature agarose (Burmeister and Lehrach 1989) Because this method is gentle, it is extremely useful for the isolation of high-molecular-weight DNA However, it also works well for smaller DNA fragments In brief

a Incubate a segment of gel containing the DNA of interest for 30 minutes at room temperature in 20 volumes of 10 mM Tris (pH 7.6), 5 mM EDTA (pH 8 0), 0 1 M NaCl

b Transfer the segment of gel to a fresh tube containing a volume of 10 mM Tris (pH 7.6), 5 mM EDTA (pH 8.0), 0 1 M NaCl approximately equal to that of the gel slice.

c Add DNAase-free agarase (available from CalBiochem). Use approximately 2 units of agarase per 100 μl of gel

d. Incubate the tube for 12–16 hours at 37°C During this time the agarose is digested to oligosaccharides If desired, the DNA solution may be used directly at this stage for ligation, restriction enzyme digestion, and transformation. Alternatively, the DNA may be purified and concentrated as described in e

e Extract the solution twice with phenol After the second extraction, transfer the aqueous phase to a fresh tube and add 2 volumes of TE (pH 7 6) This step helps to prevent precipitation of oligosaccharides Add 0 05 volume of 5 M NaCl followed by 2 volumes of ethanol Incubate the tube at 0°C for 15 minutes and then collect the precipitate by centrifugation at 12,000*g* for 15 minutes at 4°C Carefully remove the ethanol and add 0 5 ml of 70% ethanol at room temperature Vortex the tube and then recentrifuge as described above Remove the supernatant and allow the pellet of DNA to dry at room temperature Dissolve the DNA in an appropriate volume of TE (pH 7.6)

Purification of DNA Recovered from Agarose Gels

PASSAGE THROUGH DEAE-SEPHACEL

In this method, the negatively charged DNA is bound to an anionic matrix and the contaminants are washed through the column. The DNA is then eluted from the matrix by raising the ionic strength of the buffer. Conventional chromatographic resins such as DEAE-Sephadex or DEAE-Sephacel work well in this method. Alternatively, disposable columns of reversed-phase resins can be purchased from several manufacturers (e.g., Schleicher and Schuell Elutip-d). If commercial columns are used, follow the instructions provided by the manufacturer.

1. Suspend DEAE-Sephacel in 20 volumes of TE (pH 7.6) containing 0.6 M NaCl. Allow the resin to settle, and then remove the supernatant by aspiration. Add another 20 volumes of TE (pH 7.6) containing 0.6 M NaCl, and gently resuspend the resin. Allow the resin to settle once more, and then remove most of the supernatant by aspiration. Store the equilibrated resin at 4°C.

2. Pack 0.6 ml (sufficient to bind 20 μg of DNA) of the DEAE-Sephacel slurry into a small column. Commercially available small, disposable columns are most convenient for this purpose. However, the barrel of a 2-ml syringe containing a small circle of filter paper to retain the DEAE-Sephacel can also be used.

3. Wash the column with each of the following:

TE (pH 7.6) containing 0.6 M NaCl	3 ml
TE (pH 7.6)	3 ml
TE (pH 7.6) containing 0.1 M NaCl	3 ml

4. Mix the DNA with gel-loading buffer (see Table 6.3, page 6.12), and load the mixture directly onto the column. Collect the flow-through and reapply it to the column.

5. Wash the column twice with 1.5 ml of TE (pH 7.6) containing 0.3 M NaCl.

6. Elute the DNA with three 0.5-ml washes of TE (pH 7.6) containing 0.6 M NaCl.

7. Extract the eluate once with phenol·chloroform.

8. Divide the aqueous phase into two microfuge tubes, and add an equal volume of isopropanol to each tube. Store the mixtures for 15 minutes at room temperature, and then recover the DNA by centrifugation at 12,000g for 10 minutes at 4°C. Wash the pellets carefully with 70% ethanol, and then redissolve the DNA in a small volume (3–5 μl) of TE (pH 7.6).

9. Check the amount and quality of the DNA fragment by gel electrophoresis:

a Mix a small aliquot (estimated to contain 50 ng) of the final preparation of the fragment with 10 µl of TE (pH 7.6), and add 2 µl of the desired gel-loading buffer (see Table 6.3)

b Load and run an agarose gel of the appropriate concentration, using as markers appropriate restriction digests of known quantities of the original DNA. The isolated fragment should comigrate with the correct fragment in the restriction digest.

c. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA. It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

EXTRACTION WITH ORGANIC SOLVENTS

This method works for most, but not all, batches of agarose Once a satisfactory batch of agarose has been identified, it should be reserved for preparative electrophoresis

1. Recover the DNA from a gel by electrophoresis in 1 × TAE electrophoresis buffer (see Table 6.2, page 6 7) and pass it over a column of packed siliconized glass wool in a siliconized pasteur pipette (see Appendix E for siliconizing glass wool and glassware). This removes small pieces of agarose.

2. Concentrate the eluate by several consecutive extractions with equal volumes of 2-butanol (see Appendix E). 2-Butanol forms the upper phase Continue extracting the lower phase until the sample has been concentrated about 20-fold or until a volume of 0 5 ml is achieved, whichever occurs first

3. Extract the concentrated eluate twice with phenol·chloroform Transfer the aqueous phase to a fresh microfuge tube If there is a significant decrease in volume during the first extraction, re-extract the organic phase once with 1/3 volume of TE (pH 7 6) and combine the two aqueous phases

4. Add 0 2 volume of 10 M ammonium acetate and 2 volumes of ethanol at 4°C Store the mixture for 10 minutes at room temperature, and recover the DNA by centrifugation at 12,000*g* for 20 minutes at room temperature in a microfuge.

   Addition of 10 μg of carrier RNA before precipitation may improve the recovery of small amounts of DNA However, before adding the RNA, make sure that the presence of RNA will not compromise subsequent reactions with the DNA (For preparation of carrier RNA, see page 6 26)

5. Resuspend the precipitate in 200 μl of TE (pH 7 6), add 25 μl of 3 M sodium acetate (pH 5.2), and reprecipitate the DNA with 2 volumes of ethanol at 4°C and recentrifuge as in step 4 Carefully rinse the pellet with 70% ethanol, and then redissolve the DNA in 3–5 μl of TE (pH 7 6)

6. Check the amount and quality of the DNA fragment by gel electrophoresis

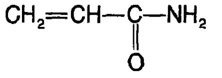
   a. Mix a small aliquot (estimated to contain 50 ng) of the final preparation of the fragment with 10 μl of TE (pH 7.6), and add 2 μl of the desired gel-loading buffer (see Table 6.3, page 6.12)

   b. Load and run an agarose gel of the appropriate concentration, using as markers appropriate restriction digests of known quantities of the original DNA The isolated fragment should comigrate with the correct fragment in the restriction digest.

c. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

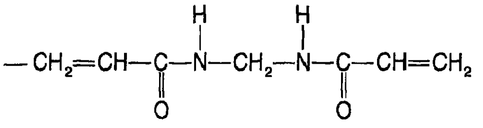
Polyacrylamide Gel Electrophoresis

Acrylamide is a monomer whose structure is



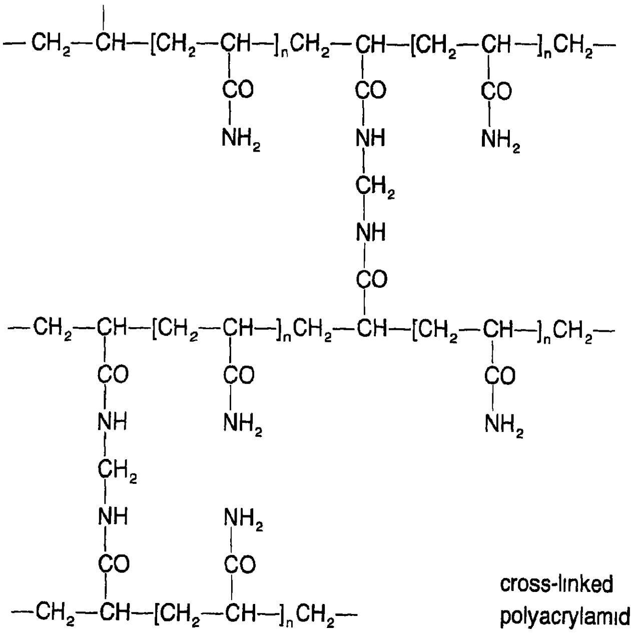
$$CH_2{=}CH{-}\underset{\underset{O}{\|}}{C}{-}NH_2$$

In the presence of free radicals, which are usually supplied by ammonium persulfate and stabilized by TEMED (*N,N,N′,N′*-tetramethylethylenediamine), a chain reaction is initiated in which monomers of acrylamide are polymerized into long chains When the bifunctional agent *N,N′*-methylenebisacrylamide is included in the polymerization reaction, the chains become cross-linked to form a gel, whose porosity is determined by the length of the chains and the degree of cross-linking



$$-CH_2{=}CH{-}\underset{\underset{O}{\|}}{C}{-}\overset{\overset{H}{|}}{N}{-}CH_2{-}\overset{\overset{H}{|}}{N}{-}\underset{\underset{O}{\|}}{C}{-}CH{=}CH_2$$

N,N′-methylenebisacrylamide



cross-linked polyacrylamide

The length of the chains is determined by the concentration of acrylamide in the polymerization reaction (between 3.5% and 20%)· 1 molecule of cross-linker is included for every 29 monomers of acrylamide. The effective range of separation in nondenaturing gels containing different concentrations of polyacrylamide is shown in Table 6.4.

Polyacrylamide gels are more of a nuisance to prepare and run than agarose gels. They are almost always poured between two glass plates that are held apart by spacers and sealed by electrical tape. In this arrangement, most of the acrylamide solution is shielded from exposure to the air, so that inhibition of polymerization by oxygen is confined to a narrow layer at the top of the gel. Polyacrylamide gels can range in length from 10 cm to 100 cm, depending on the separation required; they are invariably run in a vertical position. However, they have three major advantages over agarose gels: (1) Their resolving power is so great that they can separate molecules of DNA whose lengths differ by as little as 0.2% (i.e., 1 bp in 500 bp). (2) They can accommodate much larger quantities of DNA than agarose gels: Up to 10 μg of DNA can be applied to a single slot (1 cm × 1 mm) of a typical polyacrylamide gel without significant loss of resolution. (3) DNA recovered from polyacrylamide gels is extremely pure and can be used for the most demanding purposes (e.g., microinjection of mouse embryos).

Two types of polyacrylamide gels are in common use.

- *Nondenaturing polyacrylamide gels for the separation and purification of fragments of double-stranded DNA.* These gels are poured and run in 1 × TBE at low voltage (1–8 V/cm) to prevent denaturation of small fragments of DNA by heat generated by the passage of electric current. Most species of double-stranded DNA migrate through nondenaturing polyacrylamide gels at a rate that is approximately inversely proportional to the $\log_{10}$ of their size. However, their electrophoretic mobility is also affected by their base composition and sequence, so that DNAs of exactly the same size can differ in mobility by up to 10%. This effect is believed to be caused by kinks that form at specific sequences in double-stranded DNA. Because it is impossible to know whether or not the migration of an unknown DNA is anomalous, electrophoresis through nondenaturing polyacrylamide gels cannot be used to determine the size of double-stranded DNAs.

- *Denaturing polyacrylamide gels for the separation and purification of single-stranded fragments of DNA.* These gels are polymerized in the presence of an agent (urea or, less frequently, formamide) that suppresses base pairing in nucleic acids. (Alkali cannot be used as a denaturing agent

TABLE 6.4 Effective Range of Separation of DNAs in Polyacrylamide Gels

Acrylamide (% [w/v])[a]	Effective range of separation (bp)	Xylene cyanol FF[b]	Bromophenol blue[b]
3.5	1000–2000	460	100
5.0	80–500	260	65
8.0	60–400	160	45
12.0	40–200	70	20
15.0	25–150	60	15
20.0	6–100	45	12

[a] *N,N′*-methylenebisacrylamide is included at 1/30th the concentration of acrylamide.

[b] The numbers given are the approximate sizes (in nucleotide pairs) of fragments of double-stranded DNA with which the dye comigrates.

because it deaminates acrylamide, and methylmercuric hydroxide cannot be used because it inhibits polymerization.) Denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence. Among the uses of denaturing polyacrylamide gels are the isolation of radiolabeled DNA probes, the analysis of the products of nuclease-S1 digestions, and the analysis of the products of DNA sequencing reactions. Descriptions of denaturing polyacrylamide gels are given in Chapters 11 and 13

PREPARATION OF NONDENATURING POLYACRYLAMIDE GELS

Most vertical electrophoresis tanks obtained from commercial sources are constructed to hold glass plates 20 cm × 40 cm However, it is possible to run larger or smaller gels if suitable tanks are available. Spacers vary in thickness from 0.5 mm to 2.0 mm The thicker the gel, the hotter it will become during electrophoresis; overheating results in "smiling" bands of DNA and other problems. Thinner gels are therefore preferred, since they produce the sharpest and flattest bands of DNA. However, it is necessary to use thicker gels when preparing large quantities of DNA (>1 μg/band). Below we describe the preparation and use of polyacrylamide gels.

1. Prepare the following solutions:

30% Acrylamide

acrylamide	29 g
N,N′-methylenebisacrylamide	1 g
H_2O to 100 ml	

Heat the solution to 37°C to dissolve the chemicals.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin The effects of acrylamide are cumulative Wear gloves and a mask when weighing powdered acrylamide and methylenebisacrylamide Wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide

Cheaper grades of acrylamide and bisacrylamide are often contaminated with metal ions Stock solutions of acrylamide can easily be purified by stirring overnight with about 0 2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration through Whatman No 1 paper

During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid This deamination reaction is catalyzed by light and alkali Check that the pH of the acrylamide solution is 7 0 or less, and store the solution in dark bottles at room temperature Fresh solutions should be prepared every few months

1 × TBE

89 mM Tris-borate
2 mM EDTA (pH 8.0)

TBE is usually made and stored as a 5 × stock solution (see Table 6.2, page 6.7). The pH of the buffer should be approximately 8.3.

TBE is used at a working strength of 1 × for polyacrylamide gel electrophoresis This is twice the strength usually used for agarose gel electrophoresis The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small and the amount of electric current passed through them is often considerable 1 × TBE is required to provide adequate buffering power

10% Ammonium persulfate

ammonium persulfate 1 g
H_2O to 10 ml

The solution may be stored at 4°C for several weeks.

2. Prepare the glass plates and spacers for pouring the gel. If necessary, clean them with KOH/methanol, which is prepared by adding ~5 g of KOH pellets to 100 ml of methanol

   Caution: Handle the KOH and the KOH/methanol solutions with great care Use gloves and a face protector.

   Then wash the glass plates and spacers in warm detergent solution and rinse them well, first in tap water and then in deionized water Hold the plates by the edges so that oils from your hands do not become deposited on the working surfaces of the plates. Rinse the plates with ethanol and set them aside to dry The glass plates must be free of grease spots to prevent air bubbles from forming in the gel

   Treat one surface of each plate with a silicone solution This prevents

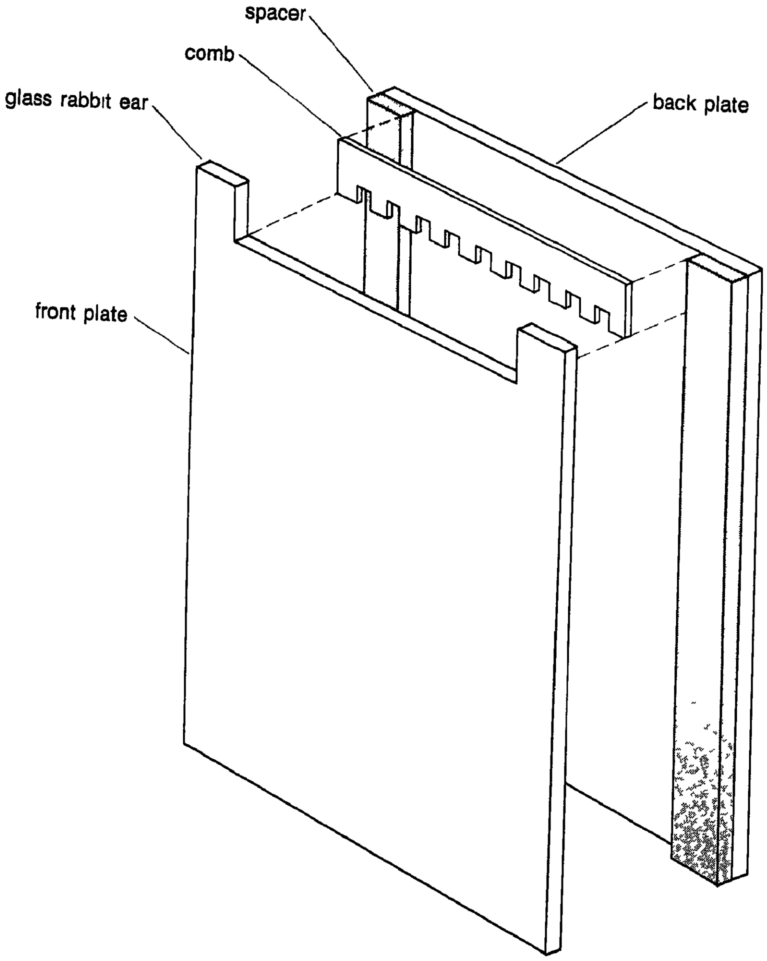


FIGURE 6.7

the gel from sticking tightly to both plates and reduces the possibility that the gel will tear when it is removed from the mold after electrophoresis is completed.

To siliconize a plate, lay the plate on a pad of paper in a fume hood and pour a small quantity of siliconizing fluid (e g , Sigmacote) onto it Wipe the fluid over the surface of the plate with a pad of Kimwipes, and then rinse the plate in deionized water. Dry the plate with a hairdryer Wear gloves during siliconization

3. There are many types of electrophoresis apparatus available commercially, and the arrangement of the glass plates and spacers differs slightly from manufacturer to manufacturer In all cases, the aim is to form a watertight seal between the plates and the spacers so that the unpolymerized gel solution does not leak out. Typically, the two plates are of slightly different size and one of them is notched. Lay the larger (or unnotched) plate flat on the bench and arrange the spacers at each side parallel to the two edges (Figure 6 7) A couple of *minute* dabs of petroleum jelly help to keep the spacer bars in position during the next steps. Lay the inner (notched) plate in position, resting on the spacer bars. Bind the entire length of the two sides and the bottom of the plates with gel-sealing tape (3M yellow electrical tape; BRL catalog no 1032ST) to make a watertight seal Take particular care with the bottom corners of the plates, since these are the places where leaks often occur

Several other methods have been used to prevent leakage of acrylamide solution from assembled gel molds, including

- Sealing the edges with agarose
- Inserting a plastic spacer into the open space at the bottom of the mold during polymerization
- Sealing the bottom of the plate with a strip of filter paper impregnated with catalyzed acrylamide (Wahls and Kingzette 1988)

We recommend that you use whichever of these methods you find to be most reliable with the particular type of gel mold that is available

4. Knowing the size of the glass plates and the thickness of the spacers, calculate the volume of acrylamide solution required (See Table 6 5 for preparation of 100 ml of solution for different gel concentrations)

5. (*Optional*) Place the required quantity of acrylamide solution in a clean sidearm flask Deaerate the solution by applying vacuum, gently at first. Swirl the flask during deaeration until no more air bubbles are released.

Deaeration of the acrylamide solution is not essential, but it does reduce the chance that air bubbles will form when thick gels (>1 mm) are poured

TABLE 6.5 Volumes of Reagents Used to Cast Polyacrylamide Gels

	Milliliters of reagents to cast gels of various concentrations (%)				
Reagents	**3.5%**	**5.0%**	**8.0%**	**12.0%**	**20.0%**
30% Acrylamide (see page 6 39)	11 6	16 6	26.6	40 0	66 6
Water	67 7	62 7	52 7	39 3	12.7
5× TBE (see Table 6.2, page 6 7)	20 0	20 0	20 0	20.0	20 0
10% Ammonium persulfate (see page 6 40)	0 7	0 7	0 7	0.7	0 7

6. Wearing gloves, perform the following manipulations over a tray so that any spilled acrylamide solution will not spread over the bench

   a. Add 35 μl of TEMED (*N,N,N',N'*-tetramethylethylenediamine) to each 100 ml of acrylamide solution. Mix the solution by swirling

   b. Draw the solution into the barrel of a 50-ml syringe Invert the syringe and expel any air that has entered the barrel. Introduce the nozzle of the syringe into the space between the two glass plates. Expel the acrylamide solution from the syringe, filling the space almost to the top Keep the remaining acrylamide solution at 4°C to reduce the rate of polymerization If the plates were clean, there should be no trapped air bubbles, and if they were sealed well, no leaks. If air bubbles form, empty the gel mold and repour the gel, after thoroughly recleaning the glass plates.

   c Lay the glass plates against a test-tube rack at an angle of approximately 10°. This decreases the chance of leakage and minimizes distortion of the gel

7. Immediately insert the appropriate comb, being careful not to allow air bubbles to become trapped under the teeth The tops of the teeth should be slightly higher than the top of the glass Clamp the comb in place with a bulldog paper clip If necessary, use the remaining acrylamide solution to fill the gel mold completely Check that no acrylamide solution is leaking from the gel mold

8. Allow the acrylamide to polymerize for 60 minutes at room temperature, adding additional acrylamide solution if the gel retracts significantly When polymerization is complete, a schlieren pattern will be visible just beneath the teeth of the comb

   Gels may be stored for 1–2 days in this state before they are used After polymerization is complete, surround the comb and the top of the gel with paper towels that have been soaked in 1 × TBE Then seal the entire gel in Saran Wrap and store at 4°C

9. Carefully remove the comb Immediately rinse out the wells with water Using a razor blade or a scalpel, remove the electrical tape from the bottom of the gel.

   It is essential to wash out the wells thoroughly as soon as the comb is removed Otherwise, small amounts of acrylamide solution trapped by the comb will polymerize in the wells, producing irregularly shaped surfaces that give rise to distorted bands of DNA

10. Attach the gel to the electrophoresis tank, using large bulldog paper clips on the sides and three-prong clamps on the shoulders The notched plate should face inward toward the buffer reservoir.

11. Fill the reservoirs of the electrophoresis tank with 1 × TBE. Use a bent

pasteur pipette or syringe needle to remove any air bubbles trapped beneath the bottom of the gel

It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA

12. Use a pasteur pipette to flush out the wells with 1 × TBE

If remnants of unpolymerized acrylamide remain in the wells, diffuse, wavy bands of DNA will be observed

13. Mix the DNA samples with the appropriate amount of 6 × gel-loading buffer (type I or type III; see Table 6.3, page 6 12). Load the mixture into the wells using a drawn-out glass micropipette or Hamilton syringe Long, disposable, and expensive micropipette tips are also sold for this purpose by several manufacturers Draw up all of the sample into the loading device, and then insert the tip of the device into the well This should be done quickly, in a single movement, since the DNA sample tends to dribble out of the loading device after the tip is immersed in the electrophoresis buffer

Usually, about 3–5 μl of DNA sample are loaded per well (0 5 cm × 0 3 cm × 1 mm) Raise the loading device as the sample is loaded into the well The tip of the device should always be above the level of the sample in the well Do not attempt to expel all of the sample from the loading device, since this almost always produces air bubbles that blow the sample out of the well

The same device can be used to load many samples, provided it is thoroughly washed between each loading However, it is important not to take too long to complete loading the gel, otherwise, the samples will diffuse from the wells

14. Connect the electrodes to a power pack (positive electrode connected to the bottom reservoir) Nondenaturing polyacrylamide gels are usually run at voltage gradients between 1 V/cm and 8 V/cm

If electrophoresis is carried out at a higher voltage, differential heating in the center of the gel may cause bowing of the DNA bands or even melting of the strands of small DNA fragments

15. Run the gel until the marker dyes have migrated the desired distance (see Table 6 4, page 6 37) Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs Detach the glass plates, and use a scalpel or razor blade to remove the electrical tape Lay the glass plates on the bench (siliconized plate uppermost). Using a thin spatula, lift a corner of the upper glass plate Check that the gel remains attached to the lower plate Pull the upper plate smoothly away Remove the spacers

Occasionally, the gel remains attached to the siliconized plate In this case, turn over the glass plates and remove the nonsiliconized plate

16. Use one of the methods described on the following pages to detect the positions of bands of DNA in the polyacrylamide gel

DETECTION OF DNA IN POLYACRYLAMIDE GELS

Staining with Ethidium Bromide

Because polyacrylamide quenches the fluorescence of ethidium bromide, it is not possible to detect bands that contain less than about 10 ng of DNA by this method

1. Gently submerge the gel and its attached glass plate in staining solution (0 5 μg/ml ethidium bromide in 1 × TBE [see page 6 7]). Use just enough staining solution to cover the gel completely After staining for 30–45 minutes at room temperature, remove the gel, using the glass plate as a support Carefully blot excess liquid from the surface of the gel with a pad of Kimwipes Do not use absorbent paper (to which the gel will stick) Cover the gel with a piece of Saran Wrap Try to avoid creating air bubbles or folds in the Saran Wrap

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described on pages 6 16–6 17

   Try to minimize the movement of the staining solution across the surface of the gel during staining The aim is to keep the gel attached to its supporting glass plate If the gel becomes completely detached, it can usually be rescued from the staining solution on a large glass plate and transferred to a shallow water bath In most cases, the gel can then be carefully unfolded and restored to its original shape To avoid problems, some workers use a piece of plastic mesh (mesh size 1 cm, available from garden and hardware stores) to hold the gel in place during staining

2. To photograph the gel, place a piece of Saran Wrap on the surface of an ultraviolet transilluminator Invert the gel, and place it on the transilluminator Remove the glass plate, leaving the gel attached to the Saran Wrap Photograph the gel as described on page 6 19

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

Autoradiography

UNFIXED, WET GELS

If bands of radioactive DNA are to be recovered from the gel, the gel should not be fixed or dried

1. Wrap the gel, together with its supporting glass plate, in Saran Wrap. Try to avoid creating air bubbles or folds in the Saran Wrap To align the gel and the film, attach adhesive dot labels marked with radioactive ink (see page 6 21) to the surface of the Saran Wrap Cover the labels with Scotch Tape This prevents contamination of the film holder or intensifying screen with radioactive ink

2. Invert the gel and expose it to X-ray film (Kodak XAR-2 or equivalent) as follows In a darkroom, tape the sealed gel to a piece of X-ray film cut to the same size as the glass plate The plate serves as a weight to ensure good contact between the Saran Wrap and the X-ray film *Do not use a metal film cassette* (which may break the glass plate and crush the gel) Instead, wrap the gel and film in light-tight aluminum foil Expose the film for the appropriate period of time at room temperature or at −70°C with an intensifying screen (see Appendix E)

FIXED, DRIED GELS

Analytical polyacrylamide gels containing radioactive DNA may be fixed and dried before autoradiography

1. Immerse the gel, together with its attached glass plate, in 7% acetic acid for 5 minutes. Remove the gel from the fixative by carefully lifting the glass plate from the fluid

   Try to minimize the movement of fluid across the surface of the gel during fixation The aim is to keep the gel attached to its supporting glass plate If the gel becomes completely detached, it can usually be rescued from the acetic acid on a larger glass plate and transferred to a shallow water bath In most cases, the gel can then be carefully unfolded and restored to its original shape To avoid problems, some workers use a piece of plastic mesh (mesh size 1 cm, available from garden and hardware stores) to hold the gel in place during fixation

2. Rinse the gel briefly in deionized water Remove excess fluid from the surface of the gel with a pad of Kimwipes Do not use absorbent paper (to which the gel will stick)

3. Wrap the gel and glass plate in Saran Wrap, and establish an autoradiograph as described above. Alternatively, dry the gel onto a piece of Whatman 3MM paper using a commercial gel dryer Drying the gel is necessary only when the gel contains DNA labeled with weak β-emitting isotopes such as ^{35}S or such small amounts of ^{32}P-labeled DNA that long exposures (longer than 24 hours) are necessary to obtain an adequate autoradiographic image.

ISOLATION OF DNA FRAGMENTS FROM POLYACRYLAMIDE GELS

The best method to isolate DNA from polyacrylamide gels is the "crush and soak" technique originally described by Maxam and Gilbert (1977) The DNA obtained is of very high purity and is free of contaminants that inhibit enzymes or are toxic to transfected or microinjected cells Although the method is lengthy and inefficient (<30% yield for DNA fragments > 3 kb in length), it can be used to isolate both double- and single-stranded DNAs from neutral and denaturing polyacrylamide gels, respectively A more rapid technique that is used to isolate fragments of double-stranded DNA is to embed the piece of polyacrylamide containing the DNA of interest into a slit cut in an agarose gel, and then to elute the DNA onto a sliver of DEAE-cellulose membrane (Schleicher and Schuell NA-45) as described on pages 6.24–6 27

"Crush and Soak" Method

The following procedure is a modification of the technique described by Maxam and Gilbert (1977)

1. Run the polyacrylamide gel as described on pages 6 39–6 43 Locate the DNA of interest by autoradiography or by examination of ethidium-bromide-stained gels in long-wavelength ultraviolet light

2. Using a sharp scalpel or razor blade, cut out the segment of the gel containing the band of interest Do not attempt to remove the gel from the Saran Wrap before cutting, instead, cut through both the gel and the Saran Wrap, and then peel the small piece of gel containing the DNA from the Saran Wrap

   To recover a fragment of DNA identified by autoradiography, cut out from the X-ray film a small rectangle containing the autoradiographic image of the fragment Align the film over the gel and cut out the segment of polyacrylamide underneath the rectangular hole in the film

   It is a good idea to photograph or to reautoradiograph the gel after the bands of DNA have been excised This provides a permanent record of the experiment

3. Transfer the gel slice to a microfuge tube Use a disposable pipette tip to crush the slice against the wall of the tube

4. Calculate the approximate volume of the slice and add 1–2 volumes of elution buffer to the microfuge tube

   Elution buffer

   0.5 M ammonium acetate
   10 mM magnesium acetate
   1 mM EDTA (pH 8.0)
   0.1% SDS

It is convenient if the volume of elution buffer is no greater than 0 5 ml, since the eluted fragment of DNA can then be precipitated with ethanol in a single microfuge tube

5. Close the tube and incubate at 37°C on a rotating wheel or rotary platform. Small fragments of DNA (<500 bp) are eluted in 3–4 hours, larger fragments take 12–16 hours.

6. Centrifuge the sample at 12,000*g* for 1 minute at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube, being careful to avoid transferring fragments of polyacrylamide (a drawn-out pasteur pipette works well)

7. Add an additional 0.5 volume of elution buffer to the pellet of polyacrylamide, vortex briefly, and recentrifuge Combine the two supernatants

8. Remove any remaining fragments of polyacrylamide by passing the supernatant through a disposable plastic column or a syringe barrel containing a Whatman GF/C filter or packed siliconized glass wool.

9. Add 2 volumes of ethanol at 4°C and store the solution on ice for 30 minutes Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge

Even small quantities of DNA are efficiently precipitated by ethanol, perhaps because of the presence of small amounts of polyacrylamide in the eluate However, 10 μg of carrier RNA can be added before precipitation, which may improve even further the recovery of small amounts of DNA Before adding the RNA, make sure that the presence of RNA will not compromise subsequent reactions with the DNA (For preparation of carrier RNA, see page 6 26)

10. Redissolve the DNA in 200 μl of TE (pH 7 6), add 25 μl of 3 M sodium acetate (pH 5 2), and reprecipitate the DNA with 2 volumes of ethanol as described in step 9

11. Carefully rinse the pellet once with 70% ethanol, and redissolve the DNA in TE (pH 7 6) to a final volume of 10 μl

12. Check the amount and quality of the fragment by polyacrylamide gel electrophoresis

a Mix a small aliquot (estimated to contain 50 ng) of the final preparation of the fragment with 10 μl of TE (pH 7 6), and add 2 μl of the desired gel-loading buffer (see Table 6 3, page 6 12).

b Load and run a polyacrylamide gel of the appropriate concentration, using as markers appropriate restriction digests of known quantities of the original DNA. The isolated fragment should comigrate with the correct fragment in the restriction digest

c. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA. It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

Other Types of Gels

STRAND SEPARATING GELS

For some purposes (e.g , DNA sequencing by the Maxam-Gilbert procedure or hybridization of strand-specific probes to low-abundance RNAs), it is necessary to obtain separated strands of fragments of DNA In the past, such separated fragments were obtained by electrophoresis of denatured DNA through neutral agarose gels (Hayward 1972; Tibbetts et al 1974, Dunn and Sambrook 1980) or polyacrylamide gels (Maxam and Gilbert 1977, 1980; Szalay et al. 1977). The ability to generate single-stranded DNA and RNA probes from DNA cloned into the appropriate vectors and to synthesize oligonucleotides of defined sequence have made obsolete the separation of the strands of DNA by electrophoretic methods

DENATURING GRADIENT POLYACRYLAMIDE GELS

Denaturing gradient polyacrylamide gels separate fragments of DNA on the basis of their thermal denaturation In this technique, which was first described by Fischer and Lerman (1979), fragments of double-stranded DNA are driven through a polyacrylamide gel that contains increasing concentrations of a denaturant, such as urea or formamide or both At a certain concentration of denaturant, a region ("domain") of the DNA denatures and the rate of migration of the partially single-stranded fragment is significantly decreased. Because different fragments denature at different points in the gradient, two fragments of identical size can be separated from one another If the gradient of denaturant is shallow, this technique is sufficiently sensitive to allow the separation of DNA fragments that differ in only a single base pair (Fischer and Lerman 1983) An excellent description of this technique can be found in Myers et al. (1987).

PULSED-FIELD GEL ELECTROPHORESIS

All linear double-stranded DNA molecules that are larger than a certain size migrate through agarose gels at the same rate This limit of resolution is reached when the radius of gyration of the linear DNA duplex exceeds the pore size of the gel The DNA can then no longer be sieved by the gel according to its size but must instead migrate "end-on" through the matrix as if through a sinuous tube. This mode of migration is known as "reptation" Several mathematical descriptions of reptation have been published (Lerman and Frisch 1982; Lumpkin and Zimm 1982, Stellwagen 1983; Edmondson and Gray 1984; Slater and Noolandi 1985, 1986; Lalande et al 1987)

The greater the pore size of the gel, the larger the DNA that can be sieved. Thus, agarose gels cast with low concentrations of agarose (0.1%–0.2%) are capable of resolving extremely large DNA molecules (Fangman 1978; Serwer 1980) However, such gels are quite fragile and must be run very slowly. Even then, they are incapable of resolving linear DNA molecules larger than 750 kb in length. The importance of this limitation becomes apparent when it is realized that a single genetic locus (e g , the major histocompatibility locus of mammals) may occupy several thousand kilobases of DNA and that DNA molecules in the individual chromosomes of lower eukaryotes may be 7000 kb or more in length

A solution to this problem was found in 1984, when Schwartz and Cantor reported the development of pulsed-field gel electrophoresis. In this method, pulsed, alternating, orthogonal electric fields are applied to a gel Large DNA molecules become trapped in their reptation tubes every time the direction of the electric field is altered and can make no further progress through the gel until they have reoriented themselves along the new axis of the electric field. The larger the DNA molecule, the longer the time required for this realignment Molecules of DNA whose reorientation times are less than the period of the electric pulse will therefore be fractionated according to size The limit of resolution of pulsed-field gel electrophoresis depends on several factors, including

- The degree of uniformity of the two electric fields
- The absolute lengths of the electric pulses
- The ratio of the lengths of the electric pulses used to generate the two electric fields
- The angles of the two electric fields to the gel
- The relative strengths of the two electric fields

The original method described by Schwartz and Cantor (1984) was capable of resolving DNAs up to 2 megabase pairs in length However, as a consequence of improvements to the technique, resolution of DNA molecules larger than 5000 kb can now be achieved.

Design of the Apparatus

The pulsed-field gel electrophoresis apparatus originally designed by Schwartz and Cantor (1984) uses alternately pulsed, perpendicularly oriented electric fields and linear electrodes This type of apparatus and modifications that employ orthogonal electric fields generated from diode electrodes have been used successfully to obtain electrophoretic karyotypes of yeasts and parasites (see, e g., Carle and Olson 1985; Van der Ploeg et al 1985; de Jonge et al 1986; Giannini et al 1986) and to derive long-range restriction maps of particular regions of mammalian DNAs (Brown and Bird 1986; Burmeister and Lehrach 1986; Hardy et al 1986, van Ommen et al 1986; Kenwrick et al 1987, Lai et al 1987, Lindsten et al 1987) However, because the electric field generated in these apparatuses is not uniform, the speed and direction of migration of the DNA are affected by the position at which the DNA is loaded into the gel Typically, the DNA migrates in a complex path and skews toward the edge of the gel, with a consequent loss of resolution To ameliorate these problems, a number of alternative electrophoretic configurations have been developed (for review, see Lai et al. 1989). These include the periodic inversion of a single electric field (Carle et al 1986), transverse fields applied to vertical gels (Gardiner et al 1986), diode-isolated vertical electrodes (McPeek et al 1986), contour-clamped homogeneous electric fields (Chu et al 1986, Vollrath and Davis 1987), and rotating gels (Anand 1986). Although some of these modifications are no more than elaborations of the original design of Schwartz and Cantor (1984), three are conceptually different and warrant special mention

In the apparatus designed by Carle et al (1986), the perpendicular or orthogonal arrangement of two electric fields is abandoned in favor of periodic inversion of one electric field (field-inversion gel electrophoresis [FIGE]) The field is uniform in both directions, and the forward pulse is slightly longer than the reverse pulse, resulting in the movement of the DNA along a perfectly straight track To obtain maximum resolution, microprocessors are used to increase the absolute lengths of the pulses during the run while maintaining the ratio between forward and reverse pulses FIGE resolves DNA fragments up to 2000 kb in length, although the range of maximum resolution depends on the exact timing and switching intervals of the fields.

Gardiner et al (1986) use a vertical gel apparatus with platinum wire electrodes positioned on opposite sides of the gel The DNA moves first toward one set of electrodes and then toward the other as the electric fields are switched The net result of these zigzag movements is a straight line from the loading well toward the bottom of the gel. Because all of the lanes in the gel are exposed to equivalent electric fields, there is no horizontal distortion of the DNA bands As with other pulsed-field gel electrophoresis systems, the size of the molecules resolved at a given voltage is a function of the angle between the electrodes and the pulse time Typically, the electrodes are arranged at a 90° angle and 10-second pulses are used to separate molecules between 50 kb and 450 kb in length, 50- to 60-second pulses are used to fractionate molecules larger than 1000 kb in length The upper limit of resolution of the system appears to be approximately 9000 kb.

In contour-clamped homogeneous field electrophoresis (CHEF) (Chu et al. 1986; Vollrath and Davis 1987), the electric field is generated from multiple electrodes that are arranged in a square or hexagonal contour around the horizontal gel and are clamped to predetermined potentials A square contour generates electric fields that are oriented at right angles to each other; a hexagonal array of electrodes generates fields at angles of 120° or 60°, depending on the placement of the gel and the polarity of the electrodes By using a combination of low field strengths (1 3 V/cm), low concentrations of agarose (0 6%), long switching intervals (1 hour), and extended periods of electrophoresis (130 hours), it is possible to resolve DNA molecules up to 5000 kb in length (Vollrath and Davis 1987)

A number of instrumentation companies are now marketing pulsed-field gel electrophoresis apparatuses based on the designs described above However, it may be several years until a particular type of apparatus gains broad acceptance and becomes the standard Until then, we recommend that you choose the apparatus that seems to be best suited to your immediate needs and that you follow the instructions provided by the manufacturer

Staining DNA Separated by Pulsed-field Gel Electrophoresis

The standard technique used to detect DNAs separated by pulsed-field gel electrophoresis is staining with ethidium bromide (see page 6 15) However, Southern hybridization may also be used as long as the DNA is partially hydrolyzed by sequential exposure to acid and base (see Chapter 9, pages 9 34–9 35) To facilitate the detection of minor species of DNA stained with ethidium bromide, the gels are usually destained in water for extended periods of time (24–48 hours) before photography For details of methods that can be used to maximize the photographic detection of DNA stained with ethidium bromide, see page 6.15. With an exposure time of 30 seconds, it is possible to obtain good photographic images of bands that contain as little as 1 ng of DNA If necessary, even smaller amounts of DNA can be detected by staining the gel with silver as described by Porro et al (1982) and Gardiner et al (1986)

Preparation of DNA for Pulsed-field Gel Electrophoresis

To avoid shearing during extraction of large DNA molecules, cells are lysed in situ in an agarose plug (Schwartz and Cantor 1984) or a bead (Cook 1984). Most investigators now use agarose plugs because of their higher efficiency (Anand 1986) Intact cells or nuclei are resuspended in molten, low-melting-temperature agarose and solidified in blocks whose size matches the thickness of the loading slot of the gel Depending on the organism, any of a variety of substances are infused into the plug to cause lysis of the cells and removal of proteins from the DNA These procedures yield DNA that is both intact and susceptible to cleavage by restriction enzymes in situ Following digestion with the appropriate restriction enzyme(s), the plug can be loaded directly into the well of a pulsed-field gel or it can be melted before loading

Procedures to lyse mammalian cells and yeasts and to digest the liberated DNA in situ are described below

ISOLATION OF INTACT DNA FROM MAMMALIAN CELLS

1. Prepare cells or tissue samples as follows

   - *Cultured cells* Wash cells that have been growing in culture three times in ice-cold phosphate-buffered saline (PBS, see Appendix B) and resuspend them at a concentration of approximately 5×10^7 cells/ml in ice-cold L buffer

   - *Fresh tissue samples* Cut freshly excised tissue into small cubes and homogenize in ice-cold PBS in a chilled glass homogenizer with a tight-fitting pestle Remove fragments of connective tissue by filtration through two layers of cheesecloth Wash the suspended cells three times in ice-cold PBS and resuspend them at a concentration of 5×10^7 cells/ml in ice-cold L buffer

   - *Frozen tissue samples* Grind frozen tissue to a fine powder using a mortar and pestle chilled to −70°C and suspend the powdered tissue in ice-cold PBS Remove fragments of connective tissue by filtration through two layers of cheesecloth Wash the suspended cells three times in ice-cold PBS and resuspend them at a concentration of 5×10^7 cells/ml in ice-cold L buffer

   L buffer

   0 1 M EDTA (pH 8.0)
   0.01 M Tris · Cl (pH 7.6)
   0.02 M NaCl

If desired, DNA can be prepared from nuclei isolated from mammalian cells However, it is our experience that there is no advantage to be gained from preparing DNA from nuclei, since DNA isolated from intact cells is equally susceptible to cleavage by restriction enzymes

2. Prepare an equal volume of low-melting-temperature agarose (1%) in L buffer. Cool the melted agarose to 42°C

3. When the agarose has cooled to 42°C, warm the cell suspension to the same temperature. Mix the melted agarose with the suspended cells Stir the mixture with a sealed pasteur pipette to ensure that the cells are evenly dispersed throughout the agarose

4. Pipette the molten mixture into preformed Plexiglas molds (50–100 μl) *or* draw the mixture into an appropriate length of Tygon tubing (3/32-inch internal diameter) Allow the agarose to set at 4°C

5. When the agarose has set, collect the blocks from the Plexiglas molds *or* blow out the agarose from the Tygon tubing into a petri dish. Cut the cylindrical plugs into 1-cm blocks

   Each 1-cm length of agarose (45 μl) should contain approximately $1\ 5 \times 10^6$ cells and should yield approximately 5–10 μg of DNA

6. Transfer the blocks to 50 volumes of L buffer containing 1 mg/ml proteinase K and 1% Sarkosyl Incubate the blocks for 24 hours at 50°C Replace the original digestion mixture with an equal volume of fresh digestion mixture and continue incubation for a further 24 hours at 50°C

7. Incubate the blocks at 50°C in 50 volumes of TE (pH 7 6) containing 40 μg/ml PMSF (phenylmethylsulfonyl fluoride). After 1 hour, replace the original rinse buffer with an equal volume of fresh rinse buffer and continue incubation at 50°C for another hour.

   Caution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, eyes, and skin It may be fatal if inhaled, swallowed, or absorbed through the skin In case of contact, immediately flush eyes or skin with copious amounts of water Discard contaminated clothing

   PMSF is inactivated in aqueous solutions The rate of inactivation increases with pH and is faster at 25°C than at 4°C The half-life of a 20 μM aqueous solution of PMSF is about 35 minutes at pH 8 0 (James 1978) This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8 6) and stored for several hours at room temperature PMSF is usually stored as a 10 mM or 100 mM stock solution (1 74 or 17 4 mg/ml in isopropanol) at −20°C

   Blocks containing DNA can be stored for a few days in TE (pH 7 6) at 4°C Blocks are best stored for longer periods of time in 0 5 M EDTA (pH 8 0) at 4°C They can be mailed to other laboratories at room temperature in tubes filled with 0 5 M EDTA (pH 8 0)

ISOLATION OF INTACT DNA FROM YEAST

This method is a slight modification of that originally described by Schwartz and Cantor (1984)

1. Collect yeast cells growing in suspension by centrifugation at 3000*g* for 5 minutes at 4°C. Wash the cell pellet twice with 0.05 M EDTA (pH 8 0), 0 01 M Tris Cl (pH 7.6)

2. Resuspend the cells at a concentration of 3×10^{10} cells/ml in 0.05 M EDTA (pH 8 0) at 0°C

3. Prepare an equal volume of low-melting-temperature agarose (1%) in L buffer Cool the melted agarose to 42°C

   L buffer

   0.1 M EDTA (pH 8.0)
   0.01 M Tris Cl (pH 7.6)
   0.02 M NaCl

4. When the agarose has cooled to 42°C, warm the cell suspension to the same temperature Mix 5 ml of the melted agarose with 5 ml of the suspended cells Stir the mixture with a sealed pasteur pipette to ensure that the cells are evenly dispersed throughout the agarose.

5. Before the agarose sets, add 75 μl of zymolyase 5000 (Kirin Breweries, 2 mg/ml in 0 01 M sodium phosphate containing 50% glycerol). Mix the enzyme with the cell suspension

   In place of zymolyase, 75 μl of Lyticase (Sigma, 900 units/ml, 67 mg/ml) can be used

6. Pipette the molten mixture into preformed Plexiglas molds (50–100 μl) *or* draw the mixture into an appropriate length of Tygon tubing (3/32-inch internal diameter) Allow the agarose to set at 4°C

7. When the agarose has set, collect the blocks from the Plexiglas molds *or* blow out the agarose from the Tygon tubing into a petri dish Cut the cylindrical plugs into 1-cm blocks

   Each block (50 μl) should contain ~5 μg of DNA

8. Incubate the blocks for 24 hours at 37°C in 50 volumes of 0 5 M EDTA (pH 8.0), 0 01 M Tris Cl (pH 7.6) containing 1% β-mercaptoethanol. Replace the buffer with an additional 50 volumes of fresh buffer, and continue incubation for a further 24 hours at 37°C.

9. Transfer the blocks to 50 volumes of L buffer containing 1 mg/ml proteinase K and 1% Sarkosyl Incubate the blocks for 24 hours at 50°C.

Replace the original digestion mixture with an equal volume of fresh digestion mixture and continue incubation for a further 24 hours at 50°C

10. Incubate the blocks at 50°C in 50 volumes of TE (pH 7 6) containing 40 μg/ml PMSF (phenylmethylsulfonyl fluoride) After 1 hour, replace the original rinse buffer with an equal volume of fresh rinse buffer and continue incubation at 50°C for another hour

    Caution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, eyes, and skin It may be fatal if inhaled, swallowed, or absorbed through the skin In case of contact, immediately flush eyes or skin with copious amounts of water Discard contaminated clothing.

    PMSF is inactivated in aqueous solutions The rate of inactivation increases with pH and is faster at 25°C than at 4°C The half-life of a 20 μM aqueous solution of PMSF is about 35 minutes at pH 8 0 (James 1978) This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8 6) and stored for several hours at room temperature PMSF is usually stored as a 10 mM or 100 mM stock solution (1 74 or 17 4 mg/ml in isopropanol) at −20°C

    Blocks containing DNA can be stored for a few days in TE (pH 7 6) at 4°C Blocks are best stored for longer periods of time in 0 5 M EDTA (pH 8 0) at 4°C They can be mailed to other laboratories at room temperature in tubes filled with 0 5 M EDTA (pH 8 0)

RESTRICTION ENZYME DIGESTION OF DNA IN AGAROSE BLOCKS

1. If blocks were received through the mail or stored in 0.5 M EDTA (pH 8.0), incubate them in 50 volumes of TE (pH 7.6) for 30 minutes at room temperature. Then transfer the blocks to 50 volumes of fresh TE (pH 7 6) and continue incubation for a further 30 minutes

2. Transfer the blocks to individual microfuge tubes, and add 10 volumes of the appropriate 1 × restriction enzyme buffer to each tube. Incubate the tubes for 30 minutes at 4°C

3. Remove the buffer and replace it with 2–3 volumes of fresh 1 × restriction enzyme buffer Add 30–50 units of the appropriate restriction enzyme to each tube, and incubate the tubes for 12–16 hours at the optimal temperature for the restriction enzyme

4. Soak the blocks for 1 hour in 50 volumes of TE (pH 7 6) at 4°C This treatment allows any salt in the restriction enzyme buffer to diffuse from the blocks

5. Using a disposable pipette tip, push the blocks directly into the slots of a pulsed-field gel

   It is difficult to load blocks directly into the slots of some designs of pulsed-field gel electrophoresis apparatuses In this case, the blocks should be melted by heating to 65°C, and the molten agarose should then be poured down the outside of a disposable pipette tip into the gel slot

Markers for Pulsed-field Gel Electrophoresis

Because of its capacity to separate extremely large molecules of DNA, pulsed-field gel electrophoresis requires markers of extremely high molecular weight. These may be obtained by extracting DNAs from bacteriophages such as T7 (40 kb), T2 (166 kb), and G (758 kb) according to the method of Lauer et al. (1977). However, a better series of markers, which are evenly spaced over a wider range of molecular weights, can be generated by ligation of bacteriophage λ DNA into a series of nested concatemers. The following method is an adaptation of the method described by Vollrath and Davis (1987).

1. Dissolve 0.1 g of low-melting-temperature agarose in 10 ml of 20 mM $MgCl_2$, 100 mM Tris · Cl (pH 7.6). Cool the solution to 37°C.

2. Dissolve 10 μg of purified bacteriophage λ DNA (see Chapter 2) in 177.5 μl of TE (pH 7.6) and heat to 56°C for 5 minutes. This treatment denatures the cohesive termini of the bacteriophage λ DNA.

3. Cool the solution to 37°C, and rapidly add the following in the order listed.

8% polyethylene glycol (PEG 8000)	62.5 μl
0.1 M ATP	5.0 μl
0.1 M dithiothreitol	5.0 μl
bacteriophage T4 DNA ligase	0.5 Weiss unit
1% low-melting-temperature agarose solution (see step 1)	250 μl

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit as determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units as defined by New England Biolabs. 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

4. Draw the mixture into a short length of Tygon tubing (3/32-inch internal diameter), and chill the tube on ice until the agarose has completely set.

5. Blow the agarose plug into a sterile, disposable plastic tube containing at least 3 volumes of 1 × ligation buffer containing polyethylene glycol.

1 × Ligation buffer containing polyethylene glycol

1 mM ATP
10 mM dithiothreitol
10 mM $MgCl_2$
50 mM Tris · Cl (pH 7.5)
1% polyethylene glycol (PEG 8000)

6. Incubate the plug for 24 hours at room temperature and then transfer it to a tube containing 10 volumes of 20 mM EDTA (pH 8.0). After incubation for 1 hour more, transfer the plug to a tube containing fresh 20 mM EDTA (pH 8.0) and store at 4°C until needed

Notes

i. This procedure yields a series of concatemers that contain up to 20 tandemly arranged copies of bacteriophage λ DNA Usually, 400–600 ng of the concatenated DNA is loaded onto a single lane (1 cm in length) of a pulsed-field agarose gel

ii A rapid method to generate a series of concatemers is described in Chapter 9, page 9.24. The method given above, although slower, generates a series of concatemers that contain a more even distribution of DNA.

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7

Extraction, Purification, and Analysis of Messenger RNA from Eukaryotic Cells

A typical mammalian cell contains about 10^{-5} μg of RNA, 80–85% of which is rRNA (chiefly 28S, 18S, and 5S). Most of the remaining 15–20% consists of a variety of low-molecular-weight species (tRNAs, small nuclear RNAs, etc.) These RNAs are of defined size and sequence and can be isolated in virtually pure form by gel electrophoresis, density gradient centrifugation, or anion-exchange or high-performance liquid chromatography In contrast, mRNA, which makes up between 1% and 5% of the total cellular RNA, is heterogeneous in both size (from a few hundred bases to many kilobases in length) and sequence. However, most eukaryotic mRNAs carry at their 3′ termini a tract of polyadenylic acid residues that is generally long enough to allow mRNAs to be purified by affinity chromatography on oligo(dT)-cellulose The resulting heterogeneous population of molecules collectively encodes virtually all of the polypeptides synthesized by the cell

Extraction and Purification of RNA

CONTROLLING RIBONUCLEASE ACTIVITY

To obtain good preparations of eukaryotic mRNA, it is necessary to minimize the activity of RNAases liberated during cell lysis by using inhibitors of RNAases or methods that disrupt cells and inactivate RNAases simultaneously as discussed below. Consequently, it is also important to avoid the accidental introduction of trace amounts of RNAase from other potential sources in the laboratory A number of precautions that can be used to avoid problems with RNAases are listed below Most experienced investigators do not rigorously adhere to these precautions but may employ one or more of them when difficulties are encountered This list is intended to be used as a guide when contamination with RNAase is a problem.

Laboratory Procedures

If proper care is not taken, preparations of RNA can be contaminated with RNAases from outside sources including

- *Glassware, plasticware, and electrophoresis tanks* Sterile, disposable plasticware is essentially free of RNAases and can be used for the preparation and storage of RNA without pretreatment General laboratory glassware and plasticware, however, are often contaminated with RNAases and should be treated by baking at 180°C for 8 hours or more (glassware) or by rinsing with chloroform (plasticware) Alternatively, some workers fill beakers, tubes, and other items that are to be used for the preparation of RNA with diethyl pyrocarbonate (DEPC) (0 1% in water), which is a strong, but not absolute, inhibitor of RNAases (Fedorcsak and Ehrenberg 1966) After the DEPC-filled glassware or plasticware has been allowed to stand for 2 hours at 37°C, it is rinsed several times with sterile water and then heated to 100°C for 15 minutes (Kumar and Lindberg 1972) or autoclaved for 15 minutes at 15 lb/sq in on liquid cycle. These treatments remove traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation

  Note Carboxymethylated RNA is translated with very low efficiency in cell-free systems, however, its ability to form DNA RNA or RNA RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified

  Electrophoresis tanks used for electrophoresis of RNA should be cleaned with detergent solution, rinsed in water, dried with ethanol, and then filled with a solution of 3% H_2O_2 After 10 minutes at room temperature, the electrophoresis tank should be rinsed thoroughly with water that has been treated with 0 1% DEPC (see below)

  It is a good idea to set aside items of glassware, batches of plasticware, and electrophoresis tanks that are to be used only for experiments with RNA, to mark them distinctively, and to store them in a designated place.

  Caution: DEPC is suspected to be a carcinogen and should be handled with care

- *Contamination by workers.* A potentially major source of contamination with RNAase is the hands of the investigator. Disposable gloves should therefore be worn during the preparation of materials and solutions used for the isolation and analysis of RNA and during manipulations involving RNA Because gloves remain RNAase-free only if they do not come into contact with "dirty" glassware and surfaces, it is usually necessary to change gloves frequently when working with RNA

- *Contaminated solutions.* All solutions should be prepared using RNAase-free glassware, autoclaved water, and chemicals reserved for work with RNA that are handled with baked spatulas Wherever possible, the solutions should be treated with 0.1% DEPC for at least 12 hours at 37°C and then heated to 100°C for 15 minutes or autoclaved for 15 minutes at 15 lb/sq in on liquid cycle

  Note DEPC reacts rapidly with amines and cannot be used to treat solutions containing buffers such as Tris Reserve a fresh, unopened bottle of Tris crystals for preparation of RNAase-free solutions

Inhibitors of Ribonucleases

The following three types of specific inhibitors of RNAases are widely used

- *Protein inhibitor of RNAases* Many RNAases bind tightly ($K_i \cong 3 \times 10^{10}$) to a protein isolated from human placenta (Blackburn et al 1977), forming equimolar, noncovalent complexes that are enzymatically inactive In vivo, the protein is probably an inhibitor of angiogenin, an angiogenic factor whose amino acid sequence and predicted tertiary structure are similar to those of pancreatic RNAase (Kurachi et al 1985, Strydom et al 1985) The inhibitor, which is sold by several manufacturers under various trade names, should be stored at −20°C in 50% glycerol solutions containing 5 mM dithiothreitol Preparations of the inhibitor that have been frozen and thawed several times or stored under oxidizing conditions should not be used; these treatments may denature the protein and release bound RNAases The inhibitor is therefore not used when denaturing agents are used to lyse mammalian cells in the initial stages of extraction of RNA However, it should be included when more gentle methods of lysis are used and should be present at all stages during the subsequent purification of RNA Fresh inhibitor should be added several times during the purification procedure, since it is removed by extraction with phenol. The inhibitor requires sulfhydryl reagents for maximal activity and does not interfere with reverse transcription (de Martynoff et al 1980) or cell-free translation of mRNA (Scheele and Blackburn 1979).

- *Vanadyl-ribonucleoside complexes.* The complexes formed between the oxovanadium IV ion and any of the four ribonucleosides are transition-state analogs that bind to many RNAases and inhibit their activity almost completely (Berger and Birkenmeier 1979). The four vanadyl-ribonucleoside complexes are added to intact cells and used at a concentration of 10 mM during all stages of RNA extraction and purification. The resulting mRNA is isolated in a form that can be directly translated in frog oocytes and can be used as a template in some in vitro enzymatic reactions (e.g., reverse transcription of mRNA). However, vanadyl-ribonucleoside complexes strongly inhibit translation of mRNA in cell-free systems and must be removed from the mRNA by multiple extractions with phenol (equilibrated with 0.01 M Tris Cl [pH 7 8]) containing 0.1% hydroxyquinoline. Vanadyl-ribonucleoside complexes are available from several commercial suppliers.

- *Macaloid* Macaloid is a clay that has been known for many years to adsorb RNAase The clay is prepared as a slurry (see Appendix B) that is used at a final concentration of 0 015% (w/v) in buffers used to lyse cells (Favaloro et al 1980) The clay, together with its adsorbed RNAase, is removed by centrifugation at some stage during the subsequent purification of the RNA (e g , after extraction with phenol).

Methods That Disrupt Cells and Inactivate Ribonucleases Simultaneously

Proteins dissolve readily in solutions of potent denaturing agents such as guanidine HCl and guanidinium thiocyanate (Cox 1968) Cellular structures disintegrate and nucleoproteins dissociate from nucleic acids as protein secondary structure is lost RNAases can recover activity after many forms of treatment (such as boiling) but are inactivated by 4 M guanidinium thiocyanate and reducing agents such as β-mercaptoethanol (Sela et al 1957). This combination of reagents can therefore be used to isolate intact RNA from tissues, such as the pancreas, that are rich in RNAase (Chirgwin et al 1979)

The protocols presented below use inhibitors of RNAase and/or methods that lead to the rapid inactivation of RNAases for the isolation of total, nuclear, and cytoplasmic RNAs from tissues and cultured cells

ISOLATION OF RNAs

Isolation of Total RNA from Mammalian Cells

This procedure is a modification of the method described by Favaloro et al (1980) for the isolation of RNA from monolayers of mammalian cells grown in tissue culture. However, it can also be used to isolate RNA from mammalian cells grown in suspension or from mammalian tissues that can be readily dispersed into single cells. This method is not suitable for extraction of RNA from solid tissue because the method used to lyse the cells (digestion with proteinase K in the presence of SDS) is slow. Endogenous RNAases therefore have time to act before they are digested by protease or inactivated by inhibitors. The lysis buffer used in the original method (Favaloro et al. 1980) contained 0.015% (w/v) of a diatomaceous earth, "Macaloid," which adsorbed and inactivated RNAases. Although Macaloid is still available (NL Chemicals), vanadyl-ribonucleoside complexes or a protein inhibitor of RNAases is now more commonly used. The advantages of the following procedure are its speed and the ability to process many samples simultaneously.

1. *Lysis of cells growing in monolayers*

   a. Remove the medium by aspiration, and wash each monolayer twice with 7 ml of ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B). Stand the plates on a bed of ice until all of the monolayers have been washed.

      The plates may be placed on an aluminum plate on top of a tray of ice.

   b. Add 0.5 ml of RNA extraction buffer per 90-mm plate. Allow the extraction buffer to spread across the surface of the plate.

      RNA extraction buffer

      0.14 M NaCl
      1.5 mM $MgCl_2$
      10 mM Tris·Cl (pH 8.6)
      0.5% Nonidet P-40 (NP-40)
      1 mM dithiothreitol
      1000 units/ml placental RNAase inhibitor *or* 20 mM vanadyl-ribonucleoside complexes

   c. Add 0.5 ml of proteinase digestion buffer. Mix the viscous lysate with a policeman, and scrape it to the edge of the plate.

      Proteinase digestion buffer

      0.2 M Tris·Cl (pH 8.0)
      25 mM EDTA (pH 8.0)
      0.3 M NaCl
      2% SDS

d. Draw the lysate into a hypodermic syringe fitted with a 21-gauge needle, and then expel it into a polypropylene tube. Repeat three or four times to shear the cellular DNA.

e Add proteinase K to a final concentration of 200 μg/ml. Mix the solution well and incubate for 30 minutes at 37°C.

Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B)

Lysis of cells growing in suspension or single-cell suspensions of tissues

a Collect the cells by centrifugation at 2000*g* for 5 minutes at 4°C Wash the cell pellet three times by resuspension in 10 volumes of ice-cold PBS lacking calcium and magnesium ions, use a wide-bore pipette to disperse the cell pellet gently, but completely, each time.

b. Estimate the volume of the packed cells, and resuspend them in 10–20 volumes of RNA extraction buffer

c Add a volume of proteinase digestion buffer equal to the volume of RNA extraction buffer added in b Mix the solution rapidly by vortexing. Draw the lysate into a hypodermic syringe fitted with a 21-gauge needle, and then expel it into a polypropylene tube Repeat three or four times to shear the cellular DNA.

d Add proteinase K to a final concentration of 200 μg/ml. Mix the solution well and incubate for 30 minutes at 37°C

Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B)

2. Remove the proteins by extracting once with an equal volume of phenol chloroform.

3. Separate the aqueous and organic phases by centrifugation at 5000*g* for 10 minutes at room temperature in a swinging-bucket rotor Transfer the aqueous phase to a fresh tube, and add 2.5 volumes of ice-cold ethanol Mix the solution well and chill to 0°C for 1 hour

4. Recover the RNA by centrifugation at 5000*g* for 10 minutes at 0°C Discard the supernatant, and wash the pellet with 70% ethanol containing 0 1 M sodium acetate (pH 5.2) Use an automatic micropipettor to remove as much of the ethanol as possible, and then allow the pellet to dry at room temperature for a few minutes

It is important not to desiccate the pellet Dried pellets of nucleic acid are very difficult to redissolve

5. Redissolve the pellet in a small volume (200 μl per 90-mm plate or 10^7 cells) of 50 mM Tris Cl (pH 7 8), 1 mM EDTA (pH 8.0).

6. Add $MgCl_2$ and dithiothreitol to final concentrations of 10 mM and 0.1 mM, respectively, and then add placental RNAase inhibitor or vanadyl-ribonucleoside complexes to a final concentration of 1000 units/ml or 10 mM, respectively.

7. Add RNAase-free pancreatic DNAase I (see Appendix B) to a final concentration of 2 μg/ml. Incubate the mixture for 60 minutes at 37°C.

8. Add EDTA and SDS to final concentrations of 10 mM and 0.2%, respectively.

9. Extract the solution once with an equal volume of phenol:chloroform.

10. Separate the aqueous and organic phases by centrifugation at 5000*g* for 10 minutes at room temperature. Transfer the aqueous phase to a fresh tube, and add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2.5 volumes of ice-cold ethanol, mix the solution well, and chill for 2 hours on ice.

11. Collect the RNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

12. Remove all of the ethanol. Stand the open tube on the bench for a few minutes to allow the last traces of ethanol to evaporate.

13. Redissolve the pellet in 200 μl of TE (pH 7.6). Add 500 μl of ethanol, and store the preparation at −70°C until it is needed.

    To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Notes

i. The concentration of the RNA can be determined by measuring the OD$_{260}$ of an aliquot of the final preparation. Recover the RNA from 10 μl of the ethanol/TE mixture (step 13) and redissolve the pellet in 400 μl of H_2O. Measure the OD$_{260}$. A solution of RNA whose OD$_{260}$ = 1 contains approximately 40 μg of RNA per milliliter. If you wish to use this RNA dilution sample, use cuvettes that have been soaked for 1 hour in concentrated HCl:methanol (1:1) and then washed extensively in water that has been treated with diethyl pyrocarbonate (DEPC) and autoclaved (see page 7.4).

   Caution: DEPC is suspected to be a carcinogen and should be handled with care.

ii. If desired, poly(A)$^+$ RNA can be purified from the preparation of total cellular RNA and freed from contaminating oligodeoxyribonucleotides by chromatography on oligo(dT)-cellulose (see pages 7.26–7.29).

iii. In some cases (e.g., when preparing RNA from cells infected with DNA viruses or from cells transfected with DNA), it is necessary to remove oligodeoxyribonucleotides from the preparation of total cellular RNA. If this is not done, problems may arise when the RNA is used to generate cDNA libraries or in primer-extension reactions. Contaminating fragments of template DNA may hybridize to the RNA and serve as primers during reverse transcription This can lead to erroneous mapping of the 5′ termini of specific mRNAs and to the generation of truncated cDNA clones

a After step 12, resuspend the pellet of nucleic acid in 200 μl of 3 M sodium acetate (pH 5 2) by repeated pipetting with an automatic micropipettor. Transfer the suspension to a fresh, sterile microfuge tube

b Centrifuge the suspension at 12,000*g* for 10 minutes at room temperature in a microfuge The RNA sediments to the bottom of the tube, while the great majority of the oligodeoxyribonucleotides remain in solution

c Discard the supernatant, and redissolve the pellet in 200 μl of TE (pH 7 6) Add 20 μl of 3 M sodium acetate (pH 5 2), mix well, and add 550 μl of ice-cold ethanol Mix the solution and chill for 30 minutes on ice Recover the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge Carefully remove the supernatant by aspiration

d Redissolve the pellet in 300 μl of TE (pH 7 6), and add 1 ml of ethanol Store the preparation at −70°C until it is needed

To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5 2) to a final concentration of 0 3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge

If necessary, vanadyl-ribonucleoside complexes can be removed by extracting the final preparation of RNA several times with phenol (equilibrated with 0 01 M Tris Cl [pH 7 8]) containing 0 1% hydroxyquinoline

iv The yield of RNA from most lines of cultured cells is 100–200 μg per 90-mm plate

Rapid Isolation of Total RNA from Mammalian Cells

In recent years, a number of rapid methods have been developed to isolate RNA from mammalian cells grown in culture. These methods fall into two classes: those that depend on differential extraction of RNA by organic solvents (e.g., phenol at acid pH; Stallcup and Washington 1983) and those that utilize differential precipitation to separate high-molecular-weight RNA from other types of nucleic acid (e g , Birnboim 1988). Both classes of method work well even with types of cultured cells (e.g , macrophages and granulocytes) that contain relatively little mRNA and have high endogenous levels of RNAase. Many samples can be handled simultaneously and there are minimal losses of RNA. The method given below is an adaptation (D Israel, unpubl) of a protocol developed by Stallcup and Washington (1983)

1. Remove the medium by aspiration, and wash each monolayer twice with 7 ml of ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B) Stand the plates on a bed of ice until all of the monolayers have been washed

   The plates can be placed on an aluminum plate on top of a tray of ice

2. Add 2 ml of 10 mM EDTA (pH 8 0), 0 5% SDS to each 90-mm plate of cells Using a policeman, scrape the lysate into a 15-ml disposable polypropylene tube (Falcon 2053 or equivalent)

3. Rinse the plate with 2 ml of 0.1 M sodium acetate (pH 5 2), 10 mM EDTA (pH 8 0). Transfer the solution to the tube containing the cell lysate

4. Add 4 ml of phenol (equilibrated with water), and mix the contents of the tube by shaking for 2 minutes at room temperature The cellular DNA precipitates as a white, stringy mass

5. Separate the phases by centrifugation at 5000 rpm for 10 minutes at 4°C in a Sorvall HB-4 or SS34 rotor (or equivalent) The DNA should form a tight cushion at the interface of the two phases

6. Using a sterile pasteur pipette, transfer the upper (aqueous) phase to a fresh tube containing 440 μl of ice-cold 1 M Tris Cl (pH 8 0) and 180 μl of 5 M NaCl.

7. Add 2 volumes of ice-cold ethanol Mix the contents of the tube and store it for at least 30 minutes at 0°C

8. Collect the RNA by centrifugation at 5000 rpm for 10 minutes at 4°C in a Sorvall HB-4 or SS34 rotor (or equivalent) Remove the ethanol, and store the tubes in an inverted position until all of the ethanol has drained away

9. Redissolve the RNA in 200 μl of ice-cold TE (pH 8.0) Transfer the solution to a sterile microfuge tube, and add 4 μl of 5 M NaCl and 500 μl of ice-cold ethanol.

10. Collect the RNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

11. Remove all of the ethanol. Stand the open tube on the bench for a few minutes to allow the last traces of ethanol to evaporate.

12. Redissolve the RNA in the desired buffer.

Notes

i. The yield of RNA from most lines of cultured cells is 100–200 μg per 90-mm plate.

ii. Several plates can be harvested simultaneously by transferring the lysate and acetate wash sequentially from plate to plate.

iii. The yield of RNA can be increased slightly by extracting the organic phase at the end of step 6 with 2 ml of a solution containing equal amounts of the EDTA/SDS lysis solution (step 2) and the sodium acetate/EDTA solution (step 3). Pool this aqueous phase with the original aqueous phase. Adjust the volumes of the reagents used in step 6 to maintain the same ratios of aqueous phase to reagents.

Isolation of Cytoplasmic RNA from Mammalian Cells

This is a convenient and well-tested procedure for purifying cytoplasmic RNA from cells grown in tissue culture The method is similar to that described previously to isolate total cellular RNA, except that the nuclei are removed by centrifugation at an early stage The procedure is rapid, many samples can be processed simultaneously, and most of the steps can be carried out at room temperature RNA prepared in this way is an excellent template for the preparation of cDNA libraries, for cell-free translation, and for primer extension and nuclease-S1 protection assays

1. *To harvest cells growing in monolayers*

a. Remove the medium by aspiration, and wash each monolayer twice with 7 ml of ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B) Stand the plates on a bed of ice until all of the monolayers have been washed

The plates may be placed on an aluminum plate on top of a tray of ice

b Using a policeman, scrape the cells into the small amount of residual PBS Transfer the cells to a microfuge tube

To harvest cells growing in suspension or single-cell suspensions of tissues.

a Collect the cells by centrifugation at 2000*g* for 5 minutes at 4°C Wash the cell pellet twice by resuspension in 10 volumes of ice-cold PBS lacking calcium and magnesium ions, use a wide-bore pipette to disperse the cell pellet gently, but completely, each time

b Estimate the volume of the packed cells, and resuspend them in 10–20 volumes of PBS Transfer 1 ml of the suspension to a microfuge tube

2. Centrifuge the cells at 12,000*g* for 30 seconds at 4°C in a microfuge Discard the supernatant, and resuspend the cell pellet in 200 μl of RNA extraction buffer. Vortex the suspension for 15 seconds, and then stand the microfuge tube on ice for 5 minutes

RNA extraction buffer

0.14 M NaCl
1 5 mM $MgCl_2$
10 mM Tris · Cl (pH 8.6)
0.5% Nonidet P-40 (NP-40)
1 mM dithiothreitol
1000 units/ml placental RNAase inhibitor *or* 20 mM vanadyl-ribonucleoside complexes

3. Centrifuge at 12,000*g* for 90 seconds at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube. Discard the pellet, which consists of unlysed cells and nuclei.

4. Add 200 μl of proteinase digestion buffer. Mix by vortexing. Add proteinase K to a final concentration of 50 μg/ml. Mix the solution well and incubate for 30 minutes at 37°C.

   Proteinase digestion buffer

   0.2 M Tris · Cl (pH 8.0)
   25 mM EDTA (pH 8.0)
   0.3 M NaCl
   2% SDS

   Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B).

   Older versions of this protocol do not call for digestion with proteinase K. However, if this step is omitted, a bulky precipitate forms at the interface of the organic and aqueous phases in step 5. The size of this precipitate is affected by the type of cells used and the conditions under which they are grown. In the worst cases, it can completely occupy the aqueous phase and make recovery of RNA very difficult.

5. Remove the proteins by extracting once with an equal volume of phenol:chloroform.

6. Separate the aqueous and organic phases by centrifugation at 5000*g* for 10 minutes at room temperature.

   It is best to centrifuge the microfuge tubes in a swinging-bucket rotor (e.g., in a Sorvall HB-4 rotor or its equivalent) rather than in a fixed-angle microfuge. In the latter case, particulate material sticks to the side of the tube and contaminates the aqueous phase.

7. Transfer the aqueous phase to a fresh microfuge tube, and add 400 μl of ice-cold isopropanol. Mix well, and chill for 30 minutes on ice.

8. Collect the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Use an automatic micropipettor fitted with a 1-ml disposable tip to remove the supernatant. Add 1 ml of 70% ethanol at room temperature, vortex briefly, and recentrifuge.

9. Carefully remove as much as possible of the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated. Proceed to step 18 or continue with steps 10–17 if necessary as noted below.

   It is important not to desiccate the pellet. Dried pellets of nucleic acid are very difficult to redissolve.

Steps 10–17 need to be carried out only when isolating cytoplasmic RNA from cells that have been transfected with DNA or from cells infected with DNA viruses. Cytoplasm prepared from such cells invariably contains a large quantity of template DNA, which must be removed before the RNA can be used for translation, northern hybridization, or synthesis of cDNA. Cytoplasm prepared from uninfected or untransfected cells generally contains only a small quantity of cellular DNA that generally does not compromise experiments with the RNA.

10. Redissolve the pellet in a small volume (200 μl per 90-mm plate or 10^7 cells) of 50 mM Tris·Cl (pH 7.8), 1 mM EDTA (pH 8.0).

11. Add $MgCl_2$ and dithiothreitol to final concentrations of 10 mM and 0.1 mM, respectively, and then add placental RNAase inhibitor or vanadyl-ribonucleoside complexes to a final concentration of 1000 units/ml or 10 mM, respectively.

12. Add RNAase-free pancreatic DNAase I (see Appendix B) to a final concentration of 2 μg/ml. Incubate for 60 minutes at 37°C.

13. Add EDTA and SDS to final concentrations of 10 mM and 0.2%, respectively.

14. Extract the solution once with an equal volume of phenol:chloroform.

15. Separate the aqueous and organic phases by centrifugation at 12,000*g* for 5 minutes at room temperature in a microfuge. Transfer the aqueous phase to a fresh microfuge tube, and add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2.5 volumes of ice-cold ethanol, mix well, and chill for at least 30 minutes on ice.

16. Collect the RNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

17. Remove all of the ethanol. Stand the open tube on the bench for a few minutes to allow the last traces of ethanol to evaporate.

18. Redissolve the pellet in 200 μl of TE (pH 7.6). Add 500 μl of ethanol, and store the preparation at −70°C until it is needed.

To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Notes

i. The concentration of the RNA can be determined by measuring the OD_{260} of an aliquot of the final preparation. Recover the RNA from 10 μl of the ethanol/TE mixture (step 18) and redissolve it in 400 μl of H_2O. Measure the OD_{260}. A solution of RNA whose $OD_{260} = 1$ contains approximately 40 μg of RNA per milliliter. If you wish to use this RNA dilution sample, use cuvettes that have been soaked for 1 hour in concentrated HCl:methanol

(1.1) and then washed extensively in water that has been treated with diethyl pyrocarbonate (DEPC) and autoclaved (see page 7.4)

Caution: DEPC is suspected to be a carcinogen and should be handled with care

ii If desired, poly(A)$^+$ RNA can be purified from the preparation of total cytoplasmic RNA and freed from contaminating oligodeoxyribonucleotides by chromatography on oligo(dT)-cellulose (see pages 7 26–7.29)

iii The yield of cytoplasmic RNA from different types of mammalian cells varies greatly—from 30 μg to 500 μg of RNA per 10^7 cells—depending on their sizes and states of differentiation The amount of RNA obtained per plate of cells is even more variable, since it is greatly influenced by the density of the cells at the time of harvest

Isolation of Total RNA from Eggs and Embryos

This simple method works very well for oocytes, fertilized eggs, and embryos of frogs, sea urchins, tunicates, worms, and flies Precipitation of the RNA with lithium chloride is helpful in removing glycoproteins and yolky components from the preparations No attempt is made to remove DNA from the preparation, since the amount of RNA obtained greatly exceeds the amount of contaminating DNA. Although these genomic DNA sequences do not interfere with hybridization or translation, they might cause complications if the RNA is to be used to generate cDNA libraries In this case, we recommend that the DNA be removed by digestion with RNAase-free pancreatic DNAase I as described on page 7.8.

1. Place the tissue in a baked glass homogenizer and remove all extraneous fluid. Fly embryos should be washed with 0 5% Triton X-100 to remove medium.

2. Add about 10 volumes of homogenization buffer, and immediately homogenize until the tissue is thoroughly dispersed

   Homogenization buffer

   50 mM NaCl
   50 mM Tris Cl (pH 7.5)
   5 mM EDTA (pH 8.0)
   0.5% SDS
   200 μg/ml proteinase K

   Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B)

3. Incubate the homogenate for 1 hour at 37°C Mix occasionally

4. Transfer the homogenate to a polypropylene tube, add an equal volume of phenol·chloroform, and vortex vigorously for 1 minute. Separate the organic and aqueous phases by centrifugation at 5000*g* for 10 minutes at room temperature in a swinging-bucket rotor

5. Transfer the aqueous (upper) phase to a fresh tube, and reextract with phenol:chloroform as described in step 4

6. Transfer the aqueous phase to a fresh tube, and add 0.1 volume of 3 M sodium acetate (pH 5.2). Mix well, and add 2.5 volumes of ice-cold ethanol. Store for 2 hours on ice.

7. Centrifuge at 5000*g* for 15 minutes at 4°C. Carefully discard the supernatant, and allow the pellet of nucleic acid to dry at room temperature. Proceed to step 11 or continue with steps 8–10 if necessary as noted below.

Many embryonic tissues contain large amounts of yolk and glycoproteins that are not removed from preparations of RNA by extraction with organic solvents In these cases, the RNA should be purified further by precipitation with LiCl (steps 8–10)

8. Redissolve the pellet in a small volume of water Add an equal volume of 8 M LiCl that has been sterilized by autoclaving, and mix well. Store at −20°C for at least 3 hours

9. Recover the RNA by centrifugation at 10,000*g* for 30 minutes at 4°C Carefully discard the supernatant

10. Wash the pellet with 70% ethanol, recentrifuge briefly, discard the supernatant, and allow the pellet of nucleic acid to dry in the air

11. Redissolve the RNA in a small volume of water Add 3 volumes of ethanol, and store the preparation at −70°C until it is needed.

    To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5 2) to a final concentration of 0 3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge

Note

The concentration of the RNA can be determined by measuring the OD_{260} of an aliquot of the final preparation Recover the RNA from 10 μl of the ethanol/water mixture (step 11) and redissolve it in 400 μl of H_2O. Measure the OD_{260} A solution of RNA whose $OD_{260} = 1$ contains approximately 40 μg of RNA per milliliter If you wish to use this RNA dilution sample, use cuvettes that have been soaked for 1 hour in concentrated HCl·methanol (1·1) and then washed extensively in water that has been treated with diethyl pyrocarbonate (DEPC) and autoclaved (see page 7 4)

Caution: DEPC is suspected to be a carcinogen and should be handled with care

Isolation of Total RNA by Extraction with Strong Denaturants

The two methods given below were devised originally to isolate RNA from cells that cannot be fractionated easily into cytoplasm and nuclei (e g , frozen fragments of tissue) or from cells that are particularly rich in RNAases (e g , pancreatic cells) (Chirgwin et al 1979) Because of their success in obtaining RNA from these "difficult" sources, the methods have become widely adopted and are now routinely used to isolate RNA from common mammalian cell lines. Both the yield and quality of RNA prepared with chaotropic agents are consistently high, and we therefore recommend that these methods be used whenever possible However, both procedures suffer from the disadvantage of being rather cumbersome and therefore are not well-suited to the isolation of RNA from large numbers of different samples of cultured cells For this purpose, one of the methods described previously might be more suitable

Tissues should be freshly harvested, weighed, cut into small pieces (1 cm^2) with a sterile scalpel, and placed directly into homogenization buffer (see below) or, if not to be used immediately, into liquid nitrogen and stored at −70°C

Cells grown in suspension should be recovered by centrifugation, washed once with phosphate-buffered saline (PBS) (see Appendix B), recentrifuged, drained well, and either used immmediately or quick-frozen in liquid nitrogen and stored at −70°C Cells grown in monolayers should be washed once with PBS, drained well, and either used immediately or quick-frozen in liquid nitrogen and stored at −70°C This can be achieved as follows

1. Make a shallow tray from aluminum foil and arrange the tissue-culture plates (with their covers on) in it Do not stack the plates on top of one another
2. Slowly pour liquid nitrogen into the tray, so that it runs underneath the bottom of the plates. After the residual liquid in the plates has frozen, quickly wrap the plates in aluminum foil and store them at −70°C

Frozen monolayers of cells stored at −70°C should be thawed directly in homogenization buffer at room temperature Frozen tissues and pellets of cells should be powdered in a chilled homogenizer, homogenization buffer should then be added to the powdered cells while they are still in the frozen state

EXTRACTION OF RNA WITH GUANIDINIUM THIOCYANATE FOLLOWED BY CENTRIFUGATION IN CESIUM CHLORIDE SOLUTIONS

This is a modification of methods described by Glišin et al. (1974) and Ullrich et al. (1977). Cells and tissues are prepared as described on pages 7.6–7.7. Guanidinium thiocyanate is used to disrupt the cells, and the resulting homogenate is then layered on a cushion of a dense solution of CsCl. The buoyant density of RNA in CsCl (>1.8 g/ml) is much greater than that of other cellular components. During centrifugation, the RNA forms a pellet on the bottom of the tube, while the DNA and protein float in the supernatant solution.

Small RNAs (e.g., 5S RNA and tRNAs), which do not sediment efficiently during centrifugation through CsCl, should not be prepared by this method.

1. *Either*

   a. To a fragment of tissue or a pellet of cells, add 5 volumes of guanidinium thiocyanate homogenization buffer.

   or

   b. To each confluent monolayer of cells, add 1 ml of guanidinium thiocyanate homogenization buffer. Allow the solution to spread across the plate until all of the cells are covered. Scrape the viscous lysate to the edge of the plate with a policeman, and transfer it to a tube for homogenization.

   Guanidinium thiocyanate homogenization buffer

   4.0 M guanidinium thiocyanate ($M_r = 118.1$)
   0.1 M Tris Cl (pH 7.5)
   1% β-mercaptoethanol

   Dissolve 50 g of guanidinium thiocyanate in 10 ml of 1 M Tris · Cl (pH 7.5), and add H_2O to 100 ml. Filter the solution through a Whatman No. 1 filter or equivalent. This solution is stable and can be stored indefinitely at room temperature. Just before use, add β-mercaptoethanol to a final concentration of 1% (0.14 M).

2. Homogenize the cell lysates with a Con-Torque tissue grinder (made by Eberbach and available from Fisher) or a Polytron homogenizer (Brinkmann) (RNAase-free) at high speed for 1–2 minutes. Homogenization shears the nuclear DNA and prevents the formation of an impenetrable mat on the top of the cushion of CsCl, which might block sedimentation of the RNA to the bottom of the centrifuge tube. Homogenization increases the yield of RNA by a factor of approximately 2.

   If commercial homogenizers are not available, the nuclear DNA can be sheared by drawing the preparation into a sterile hypodermic syringe and expelling it through a 23-gauge needle. Repeat this procedure several times until the preparation is no longer viscous.

3. After homogenization, add sodium lauryl sarcosinate to a final concentration of 0.5% and mix the suspension well. Centrifuge at 5000*g* for 10 minutes at room temperature.

   Sodium lauryl sarcosinate is used as a detergent because SDS is insoluble in concentrated salt solutions.

   The centrifugation step can usually be omitted when processing lysates of cultured cells.

4. Transfer the supernatant (or the lysate of cultured cells) to a fresh tube, and draw it into a hypodermic syringe fitted with a 23-gauge needle. Layer the samples onto a cushion of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) in a clear ultracentrifuge tube. Using a waterproof marker, mark the position of the top of the cushion on the outside of the tube. Use guanidinium thiocyanate homogenization buffer to fill the tubes and to equalize their weights.

   The CsCl/EDTA solution is made in 100-ml batches by dissolving 96.0 g of CsCl in 90 ml of 0.01 M EDTA (pH 7.5) and adding diethyl pyrocarbonate (DEPC) to a final concentration of 0.1%. Allow the solution to stand for 30 minutes, and then autoclave for 20 minutes at 15 lb/sq in. on liquid cycle. When the solution has cooled, adjust the volume to 100 ml with DEPC-treated water (see page 7.4).

   Caution: DEPC is suspected to be a carcinogen and should be handled with care.

   For convenience in later steps, the tubes should be labeled on the bottom.

5. Centrifuge at 20°C for the time and at the speed indicated in the table below.

   A swinging-bucket rotor is preferred to a fixed-angle rotor because the RNA is deposited at the bottom of the centrifuge tube rather than along the wall (where it comes into contact with the cell lysate).

   Turn off the centrifuge brake before decelerating the rotor to prevent disturbing the contents of the tube.

Rotor	Volume of homogenate (ml)	Volume of CsCl/ EDTA cushion (ml)	Time (hours)	Speed (rpm)
SW60 (7/16" × 2$\frac{3}{8}$")	1.2	3.1	12	40,000
SW41 (9/16" × 3$\frac{1}{2}$")	3.5	9.7	24	32,000
SW28 (1$\frac{1}{2}$" × 3$\frac{1}{2}$")	12.0	26.5	24	25,000

6. Take care not to disturb the gradients when removing the tubes from the centrifuge. Draw a line on the outside of each tube approximately 0.5 cm from the bottom. Using a pasteur pipette, carefully remove the fluid above the level of the cushion (upper mark on the outside of the tube). This part of the gradient contains the viscous cellular DNA, which is usually visible as a white band. With a fresh pipette, remove the fluid above the lower mark on the outside of the tube.

7. Using a red-hot razor blade held in a hemostat, cut off the bottom of the tube just above the level of the remaining fluid Place the bottom of the tube on a pad of Kimwipes, and carefully withdraw the fluid with an automatic pipettor. Invert the tube and allow any remaining fluid to drain into the pad of Kimwipes Return the tube to an upright position. Check that the pellet of RNA has not fallen out of the tube

   Depending on the amount of RNA present, the pellet may be invisible in the tube, but it can be seen easily enough if it falls onto the pad of Kimwipes Should this occur, use sterile forceps to return the pellet of RNA to the tube

8. Fill the bottom of the tube with 70% ethanol at room temperature. Invert the tube and drain off the ethanol, again checking that the pellet of RNA is not dislodged

   Washing with ethanol removes CsCl from the pellet of RNA, making it easier to dissolve

9. Allow the pellet of RNA to dry at room temperature Dissolve the pellet of RNA in the appropriate volume of TE (pH 7 6) containing 0 1% SDS by drawing the fluid repeatedly into an automatic pipettor fitted with a disposable tip

Volume of homogenate	Volume of TE (pH 7.6)/ 0.1% SDS
3 3 ml	100 μl
10 0 ml	300 μl
26 0 ml	1 0 ml

   If the RNA is difficult to dissolve, freeze and thaw the sample twice and then heat it to 45°C This usually breaks up the pellets and allows them to dissolve rapidly Alternatively, the pellets can be homogenized in the TE/SDS solution in a Con-Torque tissue grinder (made by Eberbach and available from Fisher) or a Polytron homogenizer (Brinkmann) (RNAase-free)

   Transfer the RNA solution to a microfuge tube. Rinse the bottom of the ultracentrifuge tube with 25 μl of TE (pH 7 6) and add to the microfuge tube

10. To the solution of RNA in the microfuge tube, add 150 μl of TE (pH 7 6), 30 μl of 3 M sodium acetate (pH 5 2), and 0 9 ml of ice-cold ethanol. Mix well, and store for at least 30 minutes at 0°C

11. Collect the precipitate of RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge Discard the supernatant

12. Wash the pellet with 70% ethanol, recentrifuge briefly, and allow the pellet of nucleic acid to dry in the air

13. Redissolve the RNA in a small volume of water Add 3 volumes of ethanol, and store the preparation at −70°C until it is needed

    To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0 3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Notes

i The RNA extracted from cultured cells and from most tissues is pure enough to be used for northern hybridization and nuclease-S1 analysis without further treatment However, RNA extracted from certain tissues (e.g., spleen) may be contaminated with protein, which should be removed by extraction with organic solvents The solution of RNA (step 9) is extracted once with phenol:chloroform and once with chloroform alone The RNA is then recovered from the aqueous phase by precipitation with ethanol as described in step 10. RNA isolated from liver is often contaminated with glycogen, which can be removed by precipitating the RNA with salt. Add 3 volumes of 4 M sodium acetate (pH 7.0), mix well, and store at 0°C overnight Recover the RNA by centrifugation at 8000*g* for 15 minutes at 4°C

ii. RNA isolated from cells transfected with DNA or from cells infected with DNA viruses may be contaminated with template DNA, which must be removed before the RNA can be used for translation, northern hybridization, or synthesis of cDNA (see steps 10–17, page 7 14) RNA prepared from uninfected or untransfected cells generally contains only a small quantity of cellular DNA that generally does not compromise experiments with the RNA.

EXTRACTION OF RNA WITH GUANIDINE HCl AND ORGANIC SOLVENTS

This is a modification of methods described by Strohman et al (1977) and MacDonald et al (1987) Cells and tissues are prepared as described on pages 7 6–7 7

1. *Either*

   a To a fragment of tissue or a cell pellet, add 10 volumes of guanidine HCl homogenization buffer I

   or

   b To each confluent monolayer of cells, add 1 ml of guanidine HCl homogenization buffer I Allow the solution to spread across the plate until all of the cells are covered Scrape the viscous lysate to the edge of the plate with a policeman, and transfer it to a tube for homogenization

   Guanidine HCl homogenization buffer I

   8 M guanidine HCl ($M_r = 95\ 6$)
   0 1 M sodium acetate (pH 5 2)
   5 mM dithiothreitol
   0.5% sodium lauryl sarcosinate

   Add 191 g of guanidine HCl to 8.35 ml of 3 M sodium acetate (pH 5.2) and 6.25 ml of 0 2 M dithiothreitol. Add H_2O to 237.5 ml and mix well. Add 12.5 ml of 10% sodium lauryl sarcosinate and mix by vortexing

2. Homogenize the lysates with a Con-Torque tissue grinder (made by Eberbach and available from Fisher) or a Polytron homogenizer (Brinkmann) (RNAase-free) for 1 minute at room temperature.

3. Centrifuge the homogenate at 5000*g* for 10 minutes at room temperature

   RNA may be purified from the supernatant by centrifugation through a cushion of 5 7 M CsCl, 0 01 M EDTA (pH 7 5) as described on page 7 20 or by the steps below, which involve differential precipitation of DNA and RNA

4. Transfer the supernatant to a fresh tube, and add 0 1 volume of 3 M sodium acetate (pH 5 2). Mix well Add 0 5 volume of ice-cold ethanol and mix thoroughly Store the solution for at least 2 hours at 0°C

5. Recover the nucleic acids by centrifugation at 5000*g* for 10 minutes at 0°C Discard the supernatant, and allow the pellet of nucleic acids to dry at room temperature

6. Dissolve the pellet in a small volume of guanidine HCl homogenization buffer II. Use 10–15 ml of buffer for every gram of original cells or tissue.

The pellet is often difficult to dissolve, and it may be necessary to use a homogenizer to assist in dissolution.

Guanidine HCl homogenization buffer II

8 M guanidine HCl ($M_r = 95.6$)
0.1 M sodium acetate (pH 7.0)
1 mM dithiothreitol
20 mM EDTA (pH 8.0)

Add 191 g of guanidine HCl to 8.35 ml of 3 M sodium acetate (pH 7.0), 1.25 ml of 0.2 M dithiothreitol, and 10 ml of 0.5 M EDTA (pH 8.0). Add H_2O to 250 ml and mix well.

7. Add 0 5 volume of ice-cold ethanol Immediately mix the solution Store the solution for at least 2 hours at −20°C

8. Recover the nucleic acids by centrifugation at 5000*g* for 10 minutes. Discard the supernatant, and allow the pellet of nucleic acids to dry at room temperature

9. Repeat steps 7 and 8 twice (i e , a total of three precipitations with ethanol).

10. Dissolve the pellet in a minimal volume of 0 02 M EDTA (pH 8 0) (~5 ml for every gram of original cells or tissue) as follows

a. Add approximately one half of the EDTA solution, vortex for 1–2 minutes, and centrifuge at 3000*g* for 2 minutes Remove and save the supernatant

b. Add the second half of the EDTA solution and vortex for 1–2 minutes The pellet of nucleic acids should dissolve completely

c Pool the EDTA solutions.

11. Add an equal volume of chloroform:1-butanol (4·1) and vortex

12. Centrifuge at 5000*g* for 10 minutes at room temperature. Transfer the aqueous (upper) phase to a fresh tube Repeat the extraction with organic solvents.

13. Transfer the aqueous phase to a fresh tube, and add 3 volumes of 4 M sodium acetate (pH 7.0). Store the tube for at least 1 hour at −20°C

14. Centrifuge at 5000*g* for 20 minutes at 0°C. Under these conditions, the cellular DNA remains soluble, while the RNA is precipitated.

15. Remove the supernatant, and wash the pellet once with 3 M sodium acetate (pH 7.0) at 4°C. Centrifuge at 5000*g* for 20 minutes at 0°C.

16. Remove as much as possible of the supernatant, and dissolve the pellet in a minimal volume of 0.2% SDS, 0.05 M EDTA (pH 8.0) (~1 ml per gram of cells or tissue).

If the SDS precipitates, add 0.1 N NaOH dropwise while agitating the solution until the pH reaches ~7.5.

17. Add 2 volumes of ice-cold ethanol. Store for at least 2 hours at 0°C. Recover the RNA by centrifugation at 5000*g* for 10 minutes at 4°C.

18. Wash the pellet with 70% ethanol, recentrifuge briefly, and allow the pellet of nucleic acid to dry in the air.

19. Redissolve the RNA in a small volume of water. Add 3 volumes of ethanol, and store the preparation at −70°C until it is needed.

To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Note

RNA isolated from cells transfected with DNA or from cells infected with DNA viruses may be contaminated with template DNA which must be removed before the RNA can be used for translation, northern hybridization, or synthesis of cDNA (see steps 10–17, page 7.14). RNA prepared from uninfected or untransfected cells generally contains only a small quantity of cellular DNA that generally does not compromise experiments with the RNA.

Selection of Poly(A)$^+$ RNA

In contrast to rRNAs and tRNAs, the vast majority of mRNAs of mammalian cells carry tracts of poly(A) at their 3′ termini. mRNAs can therefore be separated from the bulk of cellular RNA by affinity chromatography on oligo(dT)-cellulose (Edmonds et al. 1971; Aviv and Leder 1972). This is an essential step when preparing mRNA that is to be used as a template for the construction of cDNA libraries. Poly(A)$^+$ RNA usually yields better results than total RNA when analyzed by northern hybridization or in nuclease-S1 assays. Oligo(dT)-cellulose can be prepared as described by Gilham (1964) or obtained commercially.

1. Suspend 0.5–1.0 g of oligo(dT)-cellulose in 0.1 N NaOH.

2. Pour a column of oligo(dT)-cellulose (0.5–1.0 ml packed volume) in a sterile Dispocolumn (Bio-Rad) (or a pasteur pipette, plugged with sterile glass wool that has been treated with diethyl pyrocarbonate [DEPC] and autoclaved [see page 7.3]). Wash the column with 3 column volumes of sterile water.

   Caution: DEPC is suspected to be a carcinogen and should be handled with care.

   Up to 10 mg of total RNA can be loaded onto 1 ml of packed oligo(dT)-cellulose. If smaller quantities of total RNA are used, the amount of oligo(dT)-cellulose should be reduced accordingly to avoid loss of poly(A)$^+$ RNA both on the column and during the subsequent steps.

3. Wash the column with sterile 1 × column-loading buffer until the pH of the effluent is less than 8.0.

   1 × Column-loading buffer

   20 mM Tris · Cl (pH 7.6)
   0.5 M NaCl
   1 mM EDTA (pH 8.0)
   0.1% sodium lauryl sarcosinate

   To prepare sterile column-loading buffer, mix appropriate amounts of RNAase-free stock solutions of Tris · Cl (pH 7.6), NaCl, and EDTA (pH 8.0) (see page 7.4). Autoclave the mixture for 15 minutes at 15 lb/sq. in. on liquid cycle. Allow the solution to cool to approximately 65°C, and then add sodium lauryl sarcosinate from a 10% stock solution that has been heated to 65°C for 30 minutes. Alternatively, 0.05 M sodium citrate can be substituted for Tris · Cl, and the sodium citrate/NaCl/EDTA mixture and sodium lauryl sarcosinate can then be treated with DEPC (see page 7.4).

4. Dissolve the RNA in sterile water, and heat the solution to 65°C for 5 minutes. Cool the solution to room temperature quickly, and add an

equal amount of 2 × column-loading buffer. Apply the solution to the column, and immediately begin to collect the eluate in a sterile tube. When all of the RNA solution has entered the column, add 1 column volume of 1 × column-loading buffer and continue to collect the eluate

Heating the RNA disrupts regions of secondary structure that might involve the poly(A)$^+$ tail

5. When all of the solution has eluted, heat the collected eluate to 65°C for 5 minutes and reapply the eluate to the top of the column. Again collect the material flowing through the column

6. Wash the column with 5–10 column volumes of 1 × column-loading buffer, collecting 1-ml fractions Read the absorbance at 260 nm of each fraction collected from the column. Initially, the OD_{260} will be very high as the nonpolyadenylated RNA passes through the column The later fractions should have very little or no absorbance at 260 nm.

   In some protocols, the column is now washed with 5 column volumes of 1 × column-loading buffer containing 0 1 M NaCl However, very little or no nonpolyadenylated RNA elutes from the column during this wash, which can therefore be omitted

7. Elute the poly(A)$^+$ RNA from the oligo(dT)-cellulose with 2–3 column volumes of sterile, RNAase-free elution buffer Collect fractions equivalent in size to 1/3 to 1/2 of the column volume.

   Elution buffer

   10 mM Tris · Cl (pH 7.6)
   1 mM EDTA (pH 8.0)
   0.05% SDS

   The stock solutions of Tris · Cl and EDTA used to make elution buffer should be freshly autoclaved (15 minutes at 15 lb/sq. in. on liquid cycle) and then diluted with the appropriate amount of sterile water. The SDS should then be added from a concentrated stock solution (10% or 20%) made in sterile water. Do not attempt to sterilize elution buffer by autoclaving since it froths excessively.

8. Measure the absorbance of the solution at 260 nm, using cuvettes that have been soaked for 1 hour in concentrated HCl methanol (1 1) and then washed extensively in water that has been treated with DEPC and autoclaved (see page 7 4)

   Pool the fractions containing RNA that has eluted from the column.

   The material obtained after a single round of chromatography on oligo(dT)-cellulose usually contains approximately equal quantities of polyadenylated and nonpolyadenylated species of RNA To purify poly(A)$^+$ RNA further, heat the RNA to 65°C for 3 minutes and then cool it quickly to room temperature. Adjust the concentration of NaCl in the eluted RNA to 0.5 M, and carry out a second round of chromatography on the same column of oligo(dT)-cellulose

9. To the poly(A)$^+$ RNA eluted from the oligo(dT)-cellulose column, add 3 M sodium acetate (pH 5 2) to a final concentration of 0 3 M Mix well. Add 2 5 volumes of ice-cold ethanol, mix, and store for at least 30 minutes on ice.

10. Recover the poly(A)$^+$ RNA by centrifugation at 10,000*g* for 15 minutes at 4°C. Carefully discard the supernatant, and wash the pellet (which is often invisible) with 70% ethanol. Recentrifuge briefly, and allow the pellet of nucleic acid to dry in the air

11. Redissolve the RNA in a small volume of water Measure the absorbance of the solution at 260 nm, using cuvettes that have been soaked for 1 hour in concentrated HCl·methanol (1.1) and then washed extensively in water that has been treated with DEPC and autoclaved (see page 7 4)

12. Transfer the solution of poly(A)$^+$ RNA from the cuvettes to a polypropylene tube, add 3 volumes of ethanol, mix well, and store the preparation at −70°C until it is needed

To recover the RNA, add 3 M sodium acetate (pH 5 2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge

Notes

i A solution whose OD$_{260}$ = 1 contains approximately 40 μg of RNA per milliliter

ii The yield of poly(A)$^+$ RNA from 10^7 cultured mammalian cells should be 1–5 μg Usually between 1% and 2% of the total RNA applied to the column is recovered as poly(A)$^+$ RNA

iii Columns of oligo(dT)-cellulose can be stored at 4°C and reused many times Between uses, regenerate the column by sequential washing with NaOH, water, and column-loading buffer as described in steps 1, 2, and 3 above

iv The sodium salt of lauryl sarcosine is relatively insoluble and may therefore impede the flow of the column if the room temperature is less than 18°C This can be avoided by using LiCl instead of NaCl in the column-loading buffer.

v. When many RNA samples are to be processed, it may be more efficient to use batch affinity chromatography on oligo(dT)-cellulose. Begin at step 4 and, after dissolving the RNA in sterile water and adding 2 × column-loading buffer, add 0.3 g (dry weight) of oligo(dT)-cellulose for each 0.5 mg of RNA. Shake the suspension gently for 15 minutes at room temperature. Centrifuge the suspension at 1500*g* for 4 minutes at 15°C, discard the supernatant, and wash the oligo(dT)-cellulose four or five times with 5 ml of sterile 1 × column-loading buffer at room temperature. Elute the poly(A)$^+$ RNA with four 1-ml washes of sterile elution buffer, and continue with step 8 above

Alternatively, paper filters in which poly(U) residues have been embedded (e.g., Amersham Hybond-mAP) may be used (Wreschner and Herzberg 1984). Total cellular RNA is spotted onto the filters, which are then washed in DEPC-treated 0 1 M NaCl and 70% ethanol. Poly(A)$^+$ RNA is eluted by heating the filters to 70°C in water for 5 minutes These filters bind up to 20 μg of poly(A)$^+$ RNA per square centimeter and are extremely useful when isolating small amounts of poly(A)$^+$ RNA from many samples simultaneously Instructions for using these filters are provided by the commercial manufacturers.

vi Some protocols call for the use of poly(U)-Sephadex rather than oligo(dT)-cellulose Although both resins give excellent results, oligo(dT)-cellulose is preferred for its durability. However, poly(U)-Sephadex has a faster flow rate, which is a convenience when large volumes are to be passed through the column.

FRACTIONATION OF RNA BY SIZE IN THE PRESENCE OF METHYLMERCURIC HYDROXIDE

Two methods are used to isolate mRNA molecules of particular size electrophoresis through agarose gels and sedimentation through sucrose gradients Both methods have been used to estimate the sizes of mRNAs that code for particular proteins and to enrich populations of mRNA to be used for cDNA cloning for species of interest Electrophoresis through agarose gels gives better separation of different-size molecules of RNA, but the recovery of RNA from sucrose gradients is much more efficient. Before fractionation, the RNA is treated with methylmercuric hydroxide, a reagent that reacts primarily with the imino bonds of uridine and guanosine in RNA (Gruenwedel and Davidson 1966) Because these bonds may be involved in Watson-Crick base pairing, methylmercuric hydroxide is an effective denaturing agent that disrupts all secondary structure in RNA

The electrophoretic mobility of RNA in the presence of methylmercuric hydroxide is an inverse function of the $\log_{10}$ of its molecular weight (Bailey and Davidson 1976) Methylmercuric hydroxide can be displaced from NH groups in purines and pyrimidines and inactivated by a number of sulfhydryl compounds For example

$$CH_3HgOH + RSH \rightarrow CH_3HgSR + H_2O$$

These compounds can therefore be used to reverse the binding of methylmercuric hydroxide to RNA, which can then be translated in a cell-free protein-synthesizing system or used as a template for the synthesis of cDNA. Because methylmercuric hydroxide reacts with free radicals that are used for polymerization of acrylamide, it cannot be incorporated into polyacrylamide gels It is therefore used only in agarose gels or in sucrose density gradients

Methylmercuric hydroxide is extremely toxic It is also volatile Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood Wear gloves when handling such solutions All solid and liquid wastes should be treated as toxic materials and disposed of accordingly A thoughtful discussion that places the hazards of using alkylmercurials in reasonable perspective and describes simple and sensible precautions may be found in Junghans (1983)

Note Although fractionation of RNA by electrophoresis in the presence of methylmercuric hydroxide has been used extensively, the hazards associated with the use of methylmercury usually prompt investigators to use an alternative protocol (see pages 7 40 and 7 43)

Electrophoresis of RNA through Agarose Gels Containing Methylmercuric Hydroxide

Caution: Methylmercuric hydroxide is extremely toxic. It is also volatile Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood Wear gloves when handling such solutions. All solid and liquid wastes should be treated as toxic materials and disposed of accordingly.

1. Prepare the gel (1% agarose for RNA molecules 1 kb or larger in size; 1.4% agarose for smaller species of RNA). Dissolve the agarose in 1 × methylmercury gel-running buffer, and allow the solution to cool to 55°C before adding methylmercuric hydroxide to a final concentration of 5 mM

   1 × Methylmercury gel-running buffer

   50 mM boric acid
   5 mM $Na_2B_4O_7 \cdot 10\ H_2O$
   10 mM Na_2SO_4

   If necessary, adjust the pH to 8.1.

   Buffers that contain nitrogen bases, EDTA, or chloride ions should not be used because these compounds form complexes with methylmercuric hydroxide

   Methylmercuric hydroxide dissociates from nucleic acids when the pH is $<7\ 0$

2. Mix equal volumes of 2 × gel-loading solution and the solution of RNA (up to 10 μg may be loaded per standard 0 6-cm slot).

   2 × gel-loading solution is prepared as follows:

1 M methylmercuric hydroxide	25 μl
4 × methylmercury gel-running buffer	500 μl
100% glycerol	200 μl
H_2O	275 μl
bromophenol blue	0 2% w/v

3. Load the samples and run the gel at 5–6 V/cm for 12–16 hours

   Methylmercuric hydroxide is added to the gel but not to the methylmercury gel-running buffer The compound is uncharged and does not migrate rapidly out of the gel However, if the gel is submerged in buffer during electrophoresis, the methylmercury ions may diffuse from the gel To avoid this problem, adjust the level of the buffer so that it maintains full electrical contact with the ends of the gel but does not spill onto the surface of the gel After the samples have been loaded and the RNA has entered the gel, cover the gel with Saran Wrap to prevent drying during electrophoresis

4. After electrophoresis, RNA may be stained by incubating the gel for 30–45 minutes in 0 1 M ammonium acetate containing 0.5 μg/ml ethidium bromide. The ammonium ions convert methylmercury to a charged, nonvolatile complex and enhances binding of ethidium bromide to RNA.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E

For a description of alternative methods of staining RNA, see page 7 51

Note

Although RNA may be transferred from gels containing methylmercuric compounds to nitrocellulose filters or charged nylon membranes, this procedure is not recommended. It is less hazardous and equally effective to transfer RNA that has been fractionated through gels containing glyoxal/DMSO or formaldehyde (see pages 7 40–7 42 or 7 43–7 45, respectively)

RECOVERY OF RNA FROM AGAROSE GELS CONTAINING METHYLMERCURIC HYDROXIDE

This is a modification of the method described by Lemischka et al. (1981).

Caution: Methylmercuric hydroxide is extremely toxic It is also volatile. Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood. Wear gloves when handling such solutions All solid and liquid wastes should be treated as toxic materials and disposed of accordingly

1. Prepare and run the gel as described above, except use low-melting-temperature agarose (Wieslander 1979)

2. After electrophoresis, soak the gel in 0 1 M dithiothreitol for 30–40 minutes

   Dithiothreitol forms an insoluble complex with methylmercury, other sulfhydryl reagents, such as mercaptoethanol, form soluble complexes that retain volatility

3. Cut the gel into slices approximately 3 mm in width Stained tracks containing fragments of DNA of known size, 18S and 28S human rRNAs, or 9S rabbit β-globin mRNA may be used as molecular-weight standards The sizes of these RNAs are 6333, 2366, and 710 nucleotides, respectively Alternatively, mixtures of RNAs of known sizes can be purchased from BRL

   Dithiothreitol absorbs ultraviolet light, making detection of small amounts of RNA difficult This problem can be avoided by using as markers radioactive RNAs generated by transcription in vitro of cloned DNAs with bacteriophage-encoded DNA-dependent RNA polymerases (see Chapter 10)

4. Transfer each gel slice to a polypropylene tube, and add at least 4 volumes of 0 5 M ammonium acetate preheated to 65°C Be sure to use a large volume of ammonium acetate so that the gel slice dissolves completely Otherwise, agarose will be carried over into the aqueous phase during subsequent extraction with phenol and chloroform.

5. Heat the samples at 65°C until the agarose is completely dissolved Vortex the samples well

6. Extract the samples with phenol equilibrated with Tris Cl (pH 7 6) (see Appendix B) at room temperature Separate the phases by centrifugation at 2000g for 10 minutes at 4°C During extraction with organic solvents, agarose becomes a powder and forms a layer at the interface during centrifugation

7. Reextract the aqueous phase at least twice more with chloroform. Repeated extractions with chloroform may be required to remove the agarose completely.

8. Transfer the aqueous phase to a fresh tube, and add 0 1 volume of 3 M sodium acetate (pH 5.2) Mix well Add 3 volumes of ethanol, and store the solution for at least 1 hour at −70°C

9. Recover the RNA by centrifugation at 12,000g for 10 minutes at 4°C Carefully discard the supernatant, and wash the pellet (which is usually invisible) with 70% ethanol Recentrifuge briefly, and allow the pellet of RNA to dry in the air

10. Redissolve the RNA in a small volume (5–10 μl) of water, add 3 volumes of ethanol, mix well, and store the preparation at −70°C until it is needed.

    To recover the RNA, add 3 M sodium acetate (pH 5 2) to a final concentration of 0 3 M, mix well, and centrifuge at 12,000g for 5 minutes at 4°C in a microfuge

Note

RNA fractionated in this way can be translated in in vitro protein-synthesizing systems and is an efficient template for cDNA synthesis with reverse transcriptase

Fractionation of RNA by Centrifugation through Sucrose Gradients Containing Methylmercuric Hydroxide

This is a modification of the procedure described by Schweinfest et al. (1982).

Caution: Methylmercuric hydroxide is extremely toxic. It is also volatile. Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood. Wear gloves when handling such solutions. All solid and liquid wastes should be treated as toxic materials and disposed of accordingly.

1. Prepare one or more sucrose gradients (10–30% w/v) containing 10 mM methylmercuric hydroxide in 9/16″ × 3 1/2″ ultracentrifuge tubes (Beckman SW41 or equivalent). Up to 100 μg of RNA can be loaded on a single gradient.

   The sucrose should be dissolved in sterile water, and the solutions should then be treated overnight with 0.1% diethyl pyrocarbonate (DEPC) at 37°C and heated to 100°C for 15 minutes. After the sucrose solutions have cooled to room temperature, sterile solutions of 1 M Tris Cl (pH 7.4) and 0.5 M EDTA (pH 7.4) should be added to give final concentrations of 10 mM Tris Cl (pH 7.4) and 1 mM EDTA (pH 7.4). Finally, methylmercuric hydroxide is added to a final concentration of 10 mM.

   The apparatus used to pour the sucrose gradients and the centrifuge tubes should be treated overnight with 0.1% DEPC (or for 1 hour with 3% H_2O_2) and then rinsed thoroughly with sterile, DEPC-treated water (see page 7.4).

   Caution: DEPC is suspected to be a carcinogen and should be handled with care.

2. Dissolve the RNA (up to 100 μg) in a small volume (<100 μl/100 μg) of sterile, RNAase-free TE (pH 7.4) (see page 7.4). Add methylmercuric hydroxide to a final concentration of 20 mM, and immediately load the RNA solution onto the gradients (<100 μg/gradient).

3. Centrifuge the gradients at 34,000 rpm for 15 hours at 4°C in a Beckman SW41 rotor (or its equivalent).

4. Collect 0.3-ml fractions through a hypodermic needle inserted into the bottom of the centrifuge tube. Dilute each of the fractions with an equal volume of sterile, DEPC-treated water containing 5 mM β-mercaptoethanol. Add 60 μl of DEPC-treated 3 M sodium acetate (pH 5.2) to each fraction. Mix the samples well. Add 1.5 ml of ice-cold ethanol and mix well. Store the samples for at least 30 minutes at 0°C.

5. Centrifuge the samples at 12,000*g* for 15 minutes at 0°C. Remove and discard the supernatants (which should be treated as toxic waste). Add 1 ml of 70% ethanol, vortex briefly, and recentrifuge the samples.

6. Discard the supernatants, and allow the pellets of RNA (which are usually invisible) to dry at room temperature.

7. Redissolve the RNAs in a small volume (5–10 μl) of water, add 3 volumes of ethanol, mix well, and store the preparation at −70°C until it is needed. To recover the RNAs, add 3 M sodium acetate (pH 5.2) to a final

concentration of 0.3 M, mix well, and centrifuge at 12,000g for 5 minutes at 4°C in a microfuge

Note

RNA fractionated in this way can be translated in in vitro protein-synthesizing systems and is an efficient template for cDNA synthesis with reverse transcriptase

Analysis of RNA

A number of methods have been developed to quantitate, measure the size of, and map the 5′ and 3′ termini of specific mRNA molecules in preparations of cellular RNA These include

- *Northern hybridization (RNA blotting),* in which the size and amount of specific mRNA molecules in preparations of total or poly(A)$^+$ RNA are determined (Alwine et al 1977, 1979). The RNA is separated according to size by electrophoresis through a denaturing agarose gel and is then transferred to activated cellulose (Alwine et al 1977, Seed 1982b), nitrocellulose (Goldberg 1980, Thomas 1980, Seed 1982a), or glass or nylon membranes (Bresser and Gillespie 1983) (see below) The RNA of interest is then located by hybridization with radiolabeled DNA or RNA followed by autoradiography
- *Dot and slot hybridization,* in which an excess of radiolabeled probe is hybridized to RNA that has been immobilized on a solid support (Kafatos et al 1979; Thomas 1980; White and Bancroft 1982) Densitometric tracings of the resulting autoradiographs can allow comparative estimates of the amount of the target sequence in various preparations of RNA
- *Mapping RNA using nuclease S1 or ribonuclease,* in which the precise positions of the 5′ and 3′ termini of the mRNA and the locations of splice junctions can be rigorously determined (Berk and Sharp 1977; Weaver and Weissmann 1979) Labeled or unlabeled RNA or DNA probes derived from various segments of the genomic DNA are hybridized to mRNA, often under conditions favoring the formation of DNA.RNA hybrids (Casey and Davidson 1977) The products of the hybridization are then digested with nuclease S1 or RNAase under conditions favoring digestion of single-stranded nucleic acids only Analysis of the digestion products by gel electrophoresis yields important quantitative and qualitative information about the mRNA structure
- *Primer extension,* in which a small radiolabeled fragment of DNA is hybridized to the mRNA and used as a primer for reverse transcriptase The resulting product should extend to the extreme 5′ terminus of the mRNA, and thus the size of the product reflects the number of nucleotides from the position of the label to the 5′ terminus of the mRNA
- *Solution hybridization,* in which the absolute concentration of the sequence of interest is calculated from the rate of hybridization of a small amount of a specific radioactive probe with a known quantity of purified cellular RNA (see, e.g , Roop et al 1978; Durnam and Palmiter 1983) Alternatively, an excess of a radiolabeled probe is incubated with a known amount of RNA The concentration of the RNA of interest can then be estimated from the amount of radioactivity that becomes resistant to nuclease S1 (see, e.g , Favaloro et al. 1980; Beach and Palmiter 1981; Williams et al. 1986).

- *Filter hybridization,* in which purified cellular RNA is end-labeled with ^{32}P and hybridized to a large excess of the homologous DNA that has been immobilized on a solid support (Williams et al. 1986)

Below we describe northern hybridization Dot and slot hybridization of both crude and purified preparations of RNA are described beginning on page 7 53; nuclease-S1 and RNAase analysis of specific hybrids, beginning on pages 7 58 and 7 71, respectively; and analysis of mRNA by primer extension, beginning on page 7.79.

NORTHERN HYBRIDIZATION

Initially, northern hybridization was carried out exclusively with RNA immobilized on diazotized cellulose (diazobenzyloxymethyl [DBM]-cellulose) (Alwine et al. 1977) (see Chapter 9, pages 9.35–9.36). Subsequently, *o*-aminophenylthioether (APT)-cellulose was developed, which is easier to prepare and more stable than DBM-cellulose (Seed 1982a,b). However, the use of these activated celluloses for immobilization and hybridization of RNA was largely obviated when it was shown that RNA denatured by glyoxal and dimethyl sulfoxide (DMSO) (McMaster and Carmichael 1977), methylmercuric hydroxide (Thomas 1980, 1983), or formaldehyde (Rave et al. 1979, Goldberg 1980, Seed 1982a) binds tightly to nitrocellulose and hybridizes to radioactive probes with high efficiency (White and Bancroft 1982). The sensitivity with which RNA bound to nitrocellulose can be detected by hybridization is such that species of mRNA comprising no more than 0.001% of the mRNA can be rapidly identified and easily quantitated. Large molecules of denatured RNA (>9 kb) transfer from the gel with high efficiency, so that fragmentation of the RNA is generally unnecessary.

The attachment of denatured RNA to nitrocellulose is presumed to be noncovalent but is essentially irreversible. It is therefore possible to hybridize sequentially RNA immobilized on nitrocellulose to a series of radioactive probes without significant loss of the bound nucleic acid. Unfortunately, nitrocellulose filters are not usually durable enough to withstand more than two rounds of hybridization and washing. This difficulty can be solved by transferring the RNA to positively charged nylon membranes, which stand up well to many rounds of hybridization without loss of hybridization signal. However, the background hybridization to many types of nylon membranes is considerably higher than it is to nitrocellulose filters. We therefore recommend that nylon membranes be used only when it is known that the RNA will be hybridized to many probes sequentially. Each manufacturer provides specific instructions for the transfer of nucleic acids to their particular type of charged nylon membrane. These instructions should be followed exactly, since they presumably have been shown to yield the best results. Practical information about the use of nylon membranes can also be found in Chapter 9, pages 9.42–9.43, and in Reed and Mann (1985).

A wide variety of probes may be used to detect RNA transferred to nitrocellulose filters or nylon membranes, including double-stranded DNA labeled by nick translation, single-stranded DNA prepared by primer extension of an oligonucleotide annealed to a recombinant M13 bacteriophage, radiolabeled synthetic oligonucleotides, and RNA synthesized in vitro with prokaryotic DNA-dependent RNA polymerases (e.g., bacteriophage SP6, T7, or T3 RNA polymerase). Methods to synthesize and use these probes are discussed in Chapters 10 and 11.

Below we describe methods for electrophoresis of denatured RNA through agarose gels, for the transfer of the fractionated RNA to nitrocellulose filters and nylon membranes, and for hybridization of the immobilized RNA to radiolabeled probes. The protocol presented for northern hybridization also works well with most types of positively charged nylon membranes but may not be optimal for any particular brand.

Electrophoresis of RNA after Denaturation with Glyoxal and Dimethyl Sulfoxide

This method is adapted from that of McMaster and Carmichael (1977). Gels containing glyoxal/dimethyl sulfoxide (DMSO) are more difficult to run than those containing formaldehyde (see page 7.43): They must be run more slowly and also require that the electrophoresis buffer be circulated in order to avoid creating unacceptable H^+ gradients during electrophoresis. Although the resolving powers of the two gel systems are approximately equal (Miller 1987), the bands of RNA detected by northern hybridization are usually sharper when the RNA has been fractionated through gels containing glyoxal/DMSO.

1. In sterile microfuge tubes, mix:

6 M glyoxal	5.4 μl
DMSO	16.0 μl
0.1 M sodium phosphate (pH 7.0)	3.0 μl
RNA (up to 10 μg)	5.4 μl

Glyoxal is usually obtained as a 40% solution (6 M). Because it readily oxidizes in air, the glyoxal solution must be deionized before use by passage through a mixed-bed resin (Bio-Rad AG 501-X8) until its pH is greater than 5.0. It is then stored at −20°C in small aliquots in tightly capped tubes. Each aliquot should be used only once and then discarded. 0.1 M sodium phosphate (pH 7.0) is prepared by mixing 3.9 ml of 1 M NaH_2PO_4 with 6.1 ml of 1 M Na_2HPO_4 and 90 ml of H_2O. The sodium phosphate solution should be treated with diethyl pyrocarbonate (DEPC) and then sterilized by autoclaving (see page 7.4).

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

Up to 10 μg of RNA may be analyzed in each lane of the gel. Abundant mRNAs (0.1% or more of the mRNA population) can usually be detected by northern analysis of 10–20 μg of total cellular RNA. For detection of rare RNAs, between 0.5 and 3.0 μg of poly$(A)^+$ RNA should be applied to each lane of the gel.

2. Close the tops of the microfuge tubes, and incubate the RNA solutions for 60 minutes at 50°C. Chill the samples in ice water, and then centrifuge them for 5 seconds to deposit all of the fluid in the bottoms of the microfuge tubes.

3. While incubating the RNAs at 50°C, pour a horizontal agarose gel. For RNAs up to 1 kb in length, use 1.4% agarose; for larger RNAs, use 1% agarose. The gels are poured and run in 10 mM sodium phosphate (pH 7.0). After dissolving the agarose in the sodium phosphate, cool the solution to 70°C and add solid sodium iodoacetate to a final concentration of 10 mM (to inactivate RNAases). Cool the solution to 50°C, and then pour the gel and allow it to set for at least 30 minutes before loading the RNA.

Electrophoresis tanks used for electrophoresis of RNA should be cleaned with detergent solution, rinsed in water, dried with ethanol, and then filled with a solution of 3% H_2O_2. After 10 minutes at room temperature, the tank should be rinsed thoroughly with water that has been treated with DEPC (see page 7.4).

Because glyoxal reacts with ethidium bromide, the gels are poured and run in the absence of the dye.

4. Cool the RNA samples to 0°C, add 4 μl of sterile, DEPC-treated glyoxal/DMSO gel-loading buffer, and immediately load the samples into the wells of the gel As molecular-weight markers, use glyoxylated RNAs of known size, for example, 18S and 28S rRNAs or 9S rabbit β-globin mRNA. The sizes of these RNAs are 6333, 2366, and 710 nucleotides, respectively. Alternatively, mixtures of RNAs of known size can be purchased from BRL The markers are usually loaded into the outside lanes of the gel so that they can be cut from the gel after electrophoresis and stained with ethidium bromide If possible, leave an empty lane between the markers and the samples that are to be transferred to a nitrocellulose filter or nylon membrane

   Glyoxal/DMSO gel-loading buffer

   50% glycerol
   10 mM sodium phosphate (pH 7.0)
   0.25% bromophenol blue
   0.25% xylene cyanol FF

5. Run the gel submerged in 10 mM sodium phosphate at 3–4 V/cm. Constant recirculation of the sodium phosphate (Figure 7 1) is required to maintain the pH within acceptable limits (glyoxal dissociates from RNA at pH > 8 0) Alternatively, the sodium phosphate may be changed every 30 minutes during the run

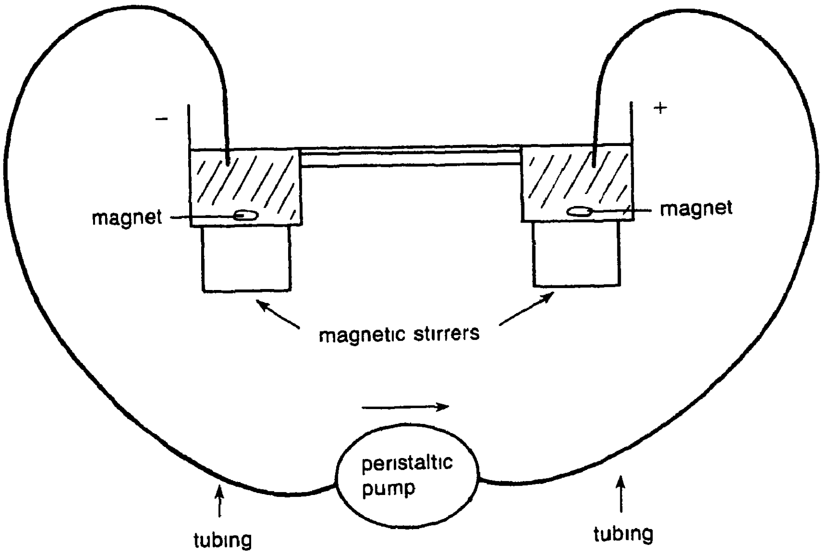

FIGURE 7.1
Recirculation of buffer during electrophoresis of RNA Either of two methods are used to maintain the pH of the electrophoresis buffer within acceptable limits· (1) The electrophoresis apparatus is placed on a pair of magnetic stirrers so that the buffer in each chamber can be mixed by means of Teflon-covered magnets. (2) A peristaltic pump is used to circulate buffer slowly from one chamber of the apparatus to the other

6. At the end of the run (when the bromophenol blue has migrated approximately 8 cm), the lanes containing the markers may be cut from the gel and stained with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30–45 minutes Align a transparent ruler with the gel, and photograph the gel and ruler by ultraviolet illumination Use the photograph to measure the distance from the loading well to each of the bands of RNA. Plot the $\log_{10}$ of the size of the fragments of RNA against the distance migrated. Use the resulting curve to calculate the sizes of the RNA species detected by hybridization after transfer from the gel to a solid support

   Cautions: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E

   Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

   For a description of alternative methods of staining RNA, see page 7 51

7. Transfer the RNA from the gel to a nitrocellulose filter as described on pages 7 46–7 48 or to a nylon membrane as described on pages 7 49–7 50

and stained with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30–45 minutes Align a transparent ruler with the gel, and photograph the gel and ruler by ultraviolet illumination. Use the photograph to measure the distance from the loading well to each of the bands of RNA. Plot the $\log_{10}$ of the size of the fragments of RNA against the distance migrated Use the resulting curve to calculate the sizes of the RNA species detected by hybridization after transfer from the gel to a solid support.

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For a description of alternative methods of staining RNA, see page 7 51

Gels containing formaldehyde are less rigid than nondenaturing agarose gels, and care must be exercised in handling them

8. Transfer the RNA from the gel to a nitrocellulose filter as described on the following pages or to a nylon membrane as described on pages 7 49–7 50

Transfer of Denatured RNA to Nitrocellulose Filters

Glyoxylated RNA may be transferred immediately after electrophoresis from agarose gels to nitrocellulose filters by capillary elution, vacuum transfer, or electroblotting (see Chapter 9, pages 9 34–9.37, for a discussion of the relative merits of these techniques) Capillary elution is carried out as described below; vacuum transfer and electroblotting should be performed according to the instructions of the manufacturer of the apparatus that is used

Although further treatment of agarose gels before transfer is unnecessary (Thomas 1980) and may be detrimental (Thomas 1983), gels containing formaldehyde must be rinsed in several changes of diethyl pyrocarbonate (DEPC)-treated water (see page 7 4) to remove the formaldehyde However, if the gel contains more than 1% agarose or is more than 0 5 cm thick or if the RNA to be analyzed is greater than 2 5 kb in length, soak the gel for 20 minutes in 0 05 N NaOH. This treatment partially hydrolyzes the RNA and improves the efficiency of transfer. Then rinse the gel in RNAase-free water and soak it for 45 minutes in 20 × SSC The gel is then placed in contact with the nitrocellulose filter or nylon membrane and the RNA is transferred to the solid support (nitrocellulose or charged nylon) in an ascending flow of buffer (Figure 7.2)

Cautions: DEPC is suspected to be a carcinogen and should be handled with care

Formaldehyde is toxic and should be handled with care in a chemical hood

Note Gels containing formaldehyde are less rigid than nondenaturing agarose gels, and care must be exercised in handling them

1. Transfer the gel to a glass baking dish, and trim away any unused areas of the gel with a razor blade Cut off the bottom left-hand corner of the gel, this serves to orient the gel during the succeeding operations

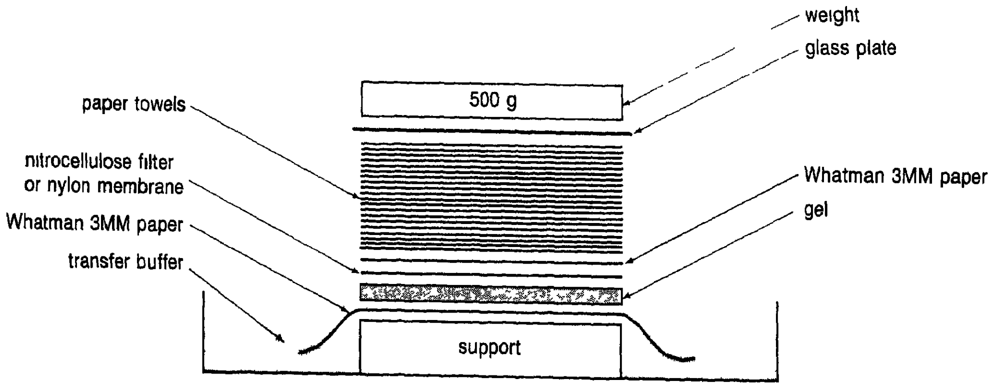


FIGURE 7.2
Capillary transfer of nucleic acids from agarose gels to solid supports Buffer is drawn from a reservoir and passes through the gel into a stack of paper towels The nucleic acid is eluted from the gel by the moving stream of buffer and is deposited on a nitrocellulose filter or nylon membrane A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system

2. Place a piece of Whatman 3MM paper on a piece of Plexiglas or a stack of glass plates to form a support that is longer and wider than the gel Place the support inside a large baking dish. Fill the dish with 20 × SSC until the level of the liquid reaches almost to the top of the support When the 3MM paper on the top of the support is thoroughly wet, smooth out all air bubbles with a glass rod

3. Using a fresh scalpel or a paper cutter, cut a piece of nitrocellulose filter (Schleicher and Schuell BA85 or equivalent) about 1 mm larger than the gel in both dimensions Use gloves and blunt-ended forceps (e.g, Millipore forceps) to handle the filter A nitrocellulose filter that has been touched by greasy hands will not wet!

4. Float the nitrocellulose filter on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the filter in 20 × SSC for at least 5 minutes Using a clean scalpel blade, cut a corner from the nitrocellulose filter to match the corner cut from the gel

   The rate at which different batches of nitrocellulose filters wet varies enormously If the filter is not saturated after floating for several minutes on water, it should be replaced with a new filter, since the transfer of RNA to an unevenly wetted filter is unreliable The original filter should not be discarded but should be autoclaved for 5 minutes between pieces of 3MM paper saturated with 2 × SSC This usually results in complete wetting of the filter The autoclaved filter, sandwiched between the autoclaved 3MM papers saturated with 2 × SSC, may be stored at 4°C in a sealed plastic bag until it is needed

5. Place the gel on the support in an inverted position so that it is centered on the wet 3MM paper Make sure that there are no air bubbles between the 3MM paper and the gel

6. Surround, but do not cover, the gel with Saran Wrap or Parafilm This serves as a barrier to prevent liquid from flowing directly from the reservoir to paper towels placed on the top of the gel If these towels are not precisely stacked, they tend to droop over the edge of the gel and may touch the support This type of short-circuiting is a major reason for inefficient transfer of RNA from the gel to the filter

7. Place the wet nitrocellulose filter on top of the gel so that the cut corners are aligned One edge of the filter should just extend over the edge of the line of slots at the top of the gel Do not move the filter once it has been applied to the surface of the gel Make sure that there are no air bubbles between the filter and the gel

8. Wet two pieces of 3MM paper (cut to exactly the same size as the gel) in 2 × SSC and place them on top of the wet nitrocellulose filter Smooth out any air bubbles with a glass rod

9. Cut a stack of paper towels (5–8 cm high) just smaller than the 3MM papers. Place the towels on the 3MM papers Put a glass plate on top of the stack and weigh it down with a 500-g weight (see Figure 7.2). The

objective is to set up a flow of liquid from the reservoir through the gel and the nitrocellulose filter, so that RNA molecules are eluted from the gel and are deposited on the nitrocellulose filter.

10. Allow transfer of RNA to proceed for 6–18 hours. As the paper towels become wet, they should be replaced

11. Remove the paper towels and the 3MM papers above the gel Turn over the gel and the nitrocellulose filter and lay them, gel side up, on a dry sheet of 3MM paper. Mark the positions of the gel slots on the filter with a very-soft-lead pencil or a ballpoint pen

12. Peel the gel from the filter and discard it Soak the filter in 6 × SSC for 5 minutes at room temperature. This removes any pieces of agarose sticking to the filter

    Remove the filter from the 6 × SSC and allow excess fluid to drain away. Place the filter flat on a paper towel to dry for at least 30 minutes at room temperature.

    To assess the efficiency of transfer of RNA, the gel may be stained for 45 minutes in a solution of ethidium bromide (0 5 μg/ml in 0 1 M ammonium acetate) and examined by ultraviolet illumination

    Cautions: Ethidium bromide is a powerful mutagen and is moderately toxic Gloves should be worn when working with solutions that contain this dye After use, these solutions should be decontaminated by one of the methods described in Appendix E

    Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

13. Place the dried filter between two pieces of 3MM paper, and bake the filter for 30 minutes to 2 hours at 80°C in a vacuum oven

    The filter will become extremely brittle and may become yellow if baked too long

    If the filter is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature

14. *For filters containing glyoxylated RNA only* Before hybridization, remove glyoxal from the RNA by washing the filter with 20 mM Tris Cl (pH 8 0) at 65°C.

Transfer of Denatured RNA to Nylon Membranes

The methods used to transfer RNA from gels to nylon membranes are similar to those used for transfer to nitrocellulose filters and include vacuum transfer, electroblotting, or capillary elution (see Chapter 9, pages 9.34–9.37, for a discussion of the relative merits of these techniques). Vacuum transfer and electroblotting should be carried out according to the instructions of the manufacturer of the apparatus that is used. Capillary elution is carried out essentially as described on pages 7.46–7.48. Gels containing formaldehyde must be rinsed in several changes of DEPC-treated water to remove the formaldehyde before transfer. Because charged nylon membranes retain nucleic acids in alkaline solution (Reed and Mann 1985), glyoxylated RNA can be eluted from agarose gels in 7.5 mN NaOH (Vrati et al. 1987). This partially hydrolyzes the RNA and thereby increases the speed and efficiency of transfer of large (>2.3 kb) RNAs. In addition, alkali removes glyoxal adducts from the mRNA (Thomas 1983), eliminating the need for postfixation stripping (step 14, page 7.48). Finally, because RNA transferred in alkaline transfer buffers becomes irreversibly fixed to the charged nylon membrane, there is no need to bake the membrane or to expose it to ultraviolet irradiation before hybridization.

The sole disadvantage of nylon membranes is a tendency to give increased levels of background hybridization, especially with RNA probes. The level of background hybridization is almost always significantly higher when the membrane has been exposed to high concentrations of alkali for extended periods of time. In many cases, this problem can be overcome by using increased amounts of blocking agents in the prehybridization and hybridization steps.

Alkaline transfer of glyoxylated RNA to charged nylon membranes is carried out essentially as described on pages 7.46–7.48, except that the transfer buffer is 7.5 mN NaOH. After transfer is completed (4.5–6.0 hours), the membranes should be rinsed briefly in 2 × SSC, 0.1% SDS and allowed to dry at room temperature.

When transfer is carried out with a neutral transfer buffer, nylon membranes must be treated to immobilize the nucleic acid after transfer is complete. RNA becomes fixed to the nylon membrane if it is thoroughly dried or if it is exposed to low doses of ultraviolet irradiation. To fix the RNA to the membrane either place the dried membrane between two pieces of 3MM paper and bake the membrane for 30 minutes to 2 hours at 80°C in a vacuum or conventional oven, or expose the side of the membrane carrying the RNA to a source of ultraviolet irradiation (254 nm). The latter method, although a nuisance to set up, is preferred because it greatly enhances the hybridization signal obtained with some brands of positively charged nylon membranes (Khandjian 1987). However, for maximum effect, it is important to make sure that the membrane is not overirradiated. The aim is to form cross-links between a small fraction of the bases in the RNA and the positively charged amine groups on the surface of the membrane (Church and Gilbert 1984). Overirradiation results in the covalent attachment of a higher proportion of thymines, with a consequent decrease in hybridization signal. Most manufacturers advise that damp nylon membranes should be exposed to a total of 1.5 J/sq. cm. and that dry membranes should be exposed to 0.15 J/sq. cm.

However, we recommend carrying out a series of preliminary experiments to determine empirically the amount of irradiation required to produce the maximum hybridization signal. In addition, the system should be recalibrated routinely.

Caution: Ultraviolet radiation is dangerous, particularly to the eyes To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light

If the membrane is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

Staining RNA Before and After Transfer to Nitrocellulose Filters

Prolonged staining with ethidium bromide is not recommended before RNA is transferred from agarose gels to nitrocellulose filters because saturation of the nucleic acid with the dye appears to reduce the efficiency of transfer. However, brief staining with ethidium bromide does not detectably inhibit transfer and has the advantage of allowing RNA to be detected both in the gel and on the filter

METHOD 1

1. After electrophoresis is completed, gels containing glyoxal/DMSO should be immersed in 10 mM sodium phosphate (pH 7 0) containing ethidium bromide (0 5 μg/ml). Gels containing formaldehyde should be washed in RNAase-free water and immersed in 20 × SSC before staining in 20 × SSC containing ethidium bromide (0 5 μg/ml)
2. After staining for 5–10 minutes at room temperature, examine and photograph the gel in ultraviolet light as described in Chapter 6, page 6 19
3. Transfer the RNA from the gel to a nitrocellulose filter as described on pages 7 46–7 48 After transfer, the stained RNA is usually visible when the filter is examined by ordinary illumination

Note

This method works only when each lane of the gel contains considerable quantities of RNA (e g , 5 μg or more of mRNA)

METHOD 2

RNA may also be stained on the nitrocellulose filter as follows A lane may be cut from the filter after it has been baked or the entire filter may be stained after hybridization and exposure to X-ray film (A Efstratiadis, unpubl)

1. Soak the dried filter in 5% acetic acid for 15 minutes at room temperature
2. Transfer the filter to a solution of 0 5 M sodium acetate (pH 5 2) and 0 04% methylene blue for 5–10 minutes at room temperature
3. Rinse the filter in water for 5–10 minutes RNAs used as molecular-weight standards should appear as sharp bands Total poly(A)$^+$ mRNA appears as a smear composed of many individual species whose sizes range between <500 bases and >5 kb The median size of mRNA is approximately 2 kb

Note

Staining with ethidium bromide is not recommended when RNA is to be transferred from gels to nylon membranes RNA may be stained with methylene blue after transfer from agarose/formaldehyde gels to certain types of nylon membrane For details of this method, see Herrin and Schmidt (1988).

Hybridization and Autoradiography

The conditions for prehybridization, hybridization, and washing of RNA immobilized on filters are essentially the same as those used for DNA These are described in detail in Chapter 9, pages 9 47–9 55 In brief

1. Prehybridize the filter for 1–2 hours in

   either (at 42°C)
   50% formamide
   5 × SSPE
   2 × Denhardt's reagent
   0.1% SDS

   or (at 68°C)
   6 × SSC
   2 × Denhardt's reagent
   0.1% SDS

2. Add the denatured radiolabeled probe directly to the prehybridization fluid To detect low-abundance mRNAs, use at least 0 1 μg of probe whose specific activity exceeds 2×10^8 cpm/μg (see Chapter 10) Continue incubation for 16–24 hours at the appropriate temperature

   It is important to remember that RNA is complementary to only one of the two DNA strands Therefore, if a single-stranded probe is utilized, it must be complementary to the RNA strand

3. Wash the filter for 20 minutes at room temperature in 1 × SSC, 0 1% SDS, followed by three washes of 20 minutes each at 68°C in 0 2 × SSC, 0 1% SDS

4. Establish an autoradiograph by exposing the filter for 24–48 hours to X-ray film (Kodak XAR-2 or equivalent) at −70°C with an intensifying screen (see Appendix E)

DOT AND SLOT HYBRIDIZATION OF RNA

Dot hybridizations were originally performed by spotting a small sample of the RNA preparation onto dry nitrocellulose, which was then dried, hybridized with a specific ^{32}P-labeled DNA or RNA probe, and exposed to X-ray film (Kafatos et al 1979) Although accurate quantitation was not feasible because of the large and variable size of the spots, it was nevertheless possible in many cases to obtain a good idea of the intensity of specific gene expression in specific tissues or cultured cells Recently, this technique has been improved in two ways

1 Filtration manifolds have been designed to accept a large number of samples and to deposit the nucleic acids onto the nitrocellulose in a fixed pattern that allows the results to be quantitated by scanning densitometry (see, e g , Brown et al 1983, Chapman et al 1983) The filtration manifold consists of a Lucite block containing a number of slots into which the samples are applied The manifold fits onto a suction platform containing a sheet of nitrocellulose onto which the samples are deposited These manifolds are available commercially (e g , Minifold II, Schleicher and Schuell)

2 A method involving slot blots has been developed (White and Bancroft 1982) to measure the concentration of a specific RNA in unfractionated cytoplasm prepared from freshly harvested or frozen cultured cells or animal tissues This improvement eliminates the tedious task of purifying RNA from large numbers of samples

Slot Hybridization of RNA

1. Wet a piece of nitrocellulose (0.45-micron pore size) briefly in water and soak it in 20× SSC for 1 hour at room temperature. Meanwhile, clean the manifold carefully with 0.1 N NaOH and then rinse it well with sterile water.

2. Place two sheets of heavy, absorbent paper, previously wetted with 20× SSC, on the top of the vacuum unit of the apparatus. Place the wet nitrocellulose on the bottom of the sample wells cut into the upper section of the manifold. Smooth away any air bubbles trapped between the upper section of the manifold and the nitrocellulose. Clamp the two parts of the manifold together, and connect the vacuum unit to a vacuum line.

3. Fill all of the slots with 10× SSC, and apply gentle suction until all of the fluid has passed through the nitrocellulose filter. Turn off the vacuum and refill the slots with 10× SSC.

4. Mix the RNA (dissolved in 10 μl of H_2O) with

100% formamide	20 μl
formaldehyde (37%)	7 μl
20× SSC	2 μl

Incubate the mixture for 15 minutes at 68°C, and then cool the samples on ice.

Caution: Formaldehyde is toxic and should be handled with care in a chemical hood.

Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

Formaldehyde (M_r = 30.03) is usually obtained as a 37% solution (12.3 M) in water. Check that the pH of the concentrated solution is greater than 4.0.

The volume of the samples containing the denatured RNA is not critical. However, the final solution should consist of
50% formamide
7% formaldehyde
1× SSC

Up to 20 μg of RNA may be applied to each slot of the manifold (John et al. 1984).

The RNA may be denatured with methylmercuric hydroxide (see page 7.30) or glyoxal (see page 7.40) instead of formaldehyde.

5. Add 2 volumes of 20× SSC to each of the samples.

6. Apply gentle suction to the manifold until the 10× SSC in the slots has passed through the nitrocellulose filter. Turn off the suction.

7. Load the samples into the slots, and then apply gentle suction After all of the samples have passed through the filter, rinse each of the slots twice with 1 ml of 10 × SSC

8. After the second rinse has passed through the filter, continue suction for 5 minutes to dry the nitrocellulose filter

9. Remove the nitrocellulose filter from the manifold, and allow it to dry completely at room temperature Bake the filter for 2 hours at 80°C in a vacuum oven

10. Hybridize the filter to a radiolabeled probe as described on page 7 52. By using single-stranded probes (either DNA or RNA) radiolabeled to a specific activity of $> 5 \times 10^8$ cpm/μg, it is possible to detect mRNAs that are present at approximately 5 copies per cell if 20 μg of total cellular RNA is applied to a single slot

To calibrate the system, add known amounts of unlabeled RNA synthesized in vitro by bacteriophage SP6 or T7 RNA polymerase from a cloned copy of the DNA probe

Slot Hybridization of Cytoplasmic RNA

This procedure is a slight modification of that described by White and Bancroft (1982). The method relies on the use of small amounts of Nonidet P-40 (NP-40) to break open the cytoplasmic membrane but not the nuclear membrane.

1. Collect 10^6–10^7 cells by centrifugation at 1000*g* for 5 minutes Resuspend the pellet in 1 ml of phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B) Transfer the cell suspension to a sterile microfuge tube, and centrifuge it at 12,000*g* for 15 seconds at 4°C in a microfuge.

2. Discard the supernatant, and resuspend the cells by brief vortexing in 45 μl of ice-cold TE (pH 7 2) Add 5 μl of a 5% solution of NP-40 Vortex briefly. Store the lysate for 5 minutes at 0°C.

   Vanadyl-ribonucleoside complexes (see pages 7 4–7 5) may be added at this stage to a final concentration of 0 1 M This is usually necessary only when cells rich in RNAases (e g , macrophages or pancreatic cells) are processed Vanadyl-ribonucleoside complexes are preferred to protein inhibitors of RNAase because they do not clog the nitrocellulose filter.

3. Add another 5 μl of 5% NP-40 Vortex the cell lysate briefly

   The amount of NP-40 required to achieve dissolution of the cytoplasmic membranes of the cell without lysis of the nuclei varies from cell line to cell line When carrying out this procedure for the first time with a new cell line, monitor the appearance of the cells during steps 1–3 by phase-contrast microscopy If necessary, adjust the concentration of NP-40 to obtain the maximum yield of free nuclei and the minimum number of unlysed cells

4. Remove the nuclei by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge

5. Transfer the supernatant to a fresh tube, taking care not to disturb the soft pellet

6. Add 30 μl of 20 × SSC to the supernatant. Vortex the solution briefly. Add 20 μl of 37% formaldehyde, vortex again, and close the top of the tube

   Caution: Formaldehyde is toxic and should be handled with care in a chemical hood.

   Formaldehyde (M_r = 30 03) is usually obtained as a 37% solution (12 3 M) in water Check that the pH of the concentrated solution is greater than 4 0

7. Incubate the tube for 15 minutes at 60°C

   The samples may be stored at −70°C at this stage, if necessary

8. Dilute 5–10 μl of the sample with 200 μl of 20 × SSC, and load it onto a nitrocellulose filter in a manifold as described on page 7.55 beginning at step 7. (For preparation of the manifold, see page 7.54, steps 1–3 and 6.)

If the filter clogs during loading, remove the insoluble material by centrifugation of the sample at 12,000*g* for 1 minute (after step 7) Be aware that this procedure can result in loss of RNA from the sample

9. Hybridize the filter to a radiolabeled probe as described on page 7 52. By using single-stranded probes (either DNA or RNA) radiolabeled to a specific activity of $>5 \times 10^8$ cpm/μg, it is possible to detect mRNAs that are present at approximately 5 copies per cell if 20 μg of total cellular RNA is applied to a single slot.

To calibrate the system, add known amounts of unlabeled RNA synthesized in vitro by bacteriophage SP6 or T7 RNA polymerase from a cloned copy of the DNA probe

MAPPING RNA WITH NUCLEASE S1

Nuclease S1 is used

- To map the location of 5′ and 3′ termini of mRNA on DNA templates
- To locate the 5′ and 3′ splice junctions in relation to sites of cleavage with restriction enzymes in cloned genes or double-stranded cDNA
- To quantitate the amount of specific classes of mRNA in RNAs extracted from tissues or cultured cells

The procedure described originally (Berk and Sharp 1977) was based on the observation of Casey and Davidson (1977) that hybridization conditions can often be established to minimize the formation of DNA·DNA hybrids while promoting the formation of DNA RNA hybrids When double-stranded DNA is denatured and incubated with mRNA under these conditions, those segments of DNA (exons) that are transcribed into mRNA form hybrids and the remainder of the DNA remains single-stranded DNA that has not formed duplexes is hydrolyzed with nuclease S1, whereas DNA that has hybridized to RNA is protected from digestion The size of the protected fragment of DNA is then determined by electrophoresis under native or denaturing conditions through agarose or acrylamide gels By using DNA fragments derived from different segments of the gene of interest, it is possible to map the termini of the mRNA and locate the positions of introns within the gene (Berk and Sharp 1977, Favaloro et al 1980) (Figure 7 3) In this type of experiment, the double-stranded DNA need not be radiolabeled The fragments of DNA that survive digestion with nuclease S1 can be detected efficiently by Southern hybridization using uniformly radiolabeled probes. Alternatively, double-stranded fragments of DNA that are radiolabeled at only one end by phosphorylation or by filling of recessed 3′ termini (see Chapter 10) may be used directly in the annealing reaction This kind of asymmetric labeling allows the polarity of the mRNA with respect to the double-stranded DNA to be established directly (Weaver and Weissmann 1979) (Figure 7 4)

Although it is simple to isolate double-stranded fragments of DNA that can be used as probes, it is not always easy or possible to establish conditions that suppress formation of DNA.DNA hybrids and yet allow annealing of DNA to RNA to proceed efficiently This problem can be avoided if single-stranded probes (either DNA or RNA) are used in the hybridization mixture Annealing can then be carried out under standard conditions because a complementary strand is not present to compete with the hybridization of the mRNA to the probe Until recently, such single-stranded probes could be obtained only by physical separation of the complementary strands of a fragment of DNA (usually by gel electrophoresis [Hayward 1972]). This method was laborious, did not work with every fragment of DNA, and hardly ever produced probes that were completely strand-specific. However, during the last few years, new methods have become available to prepare single-stranded probes of defined length and polarity from virtually any restriction fragment. These probes are now the reagents of choice for the analysis of RNA. Techniques to generate these probes are described in Chapter 10.

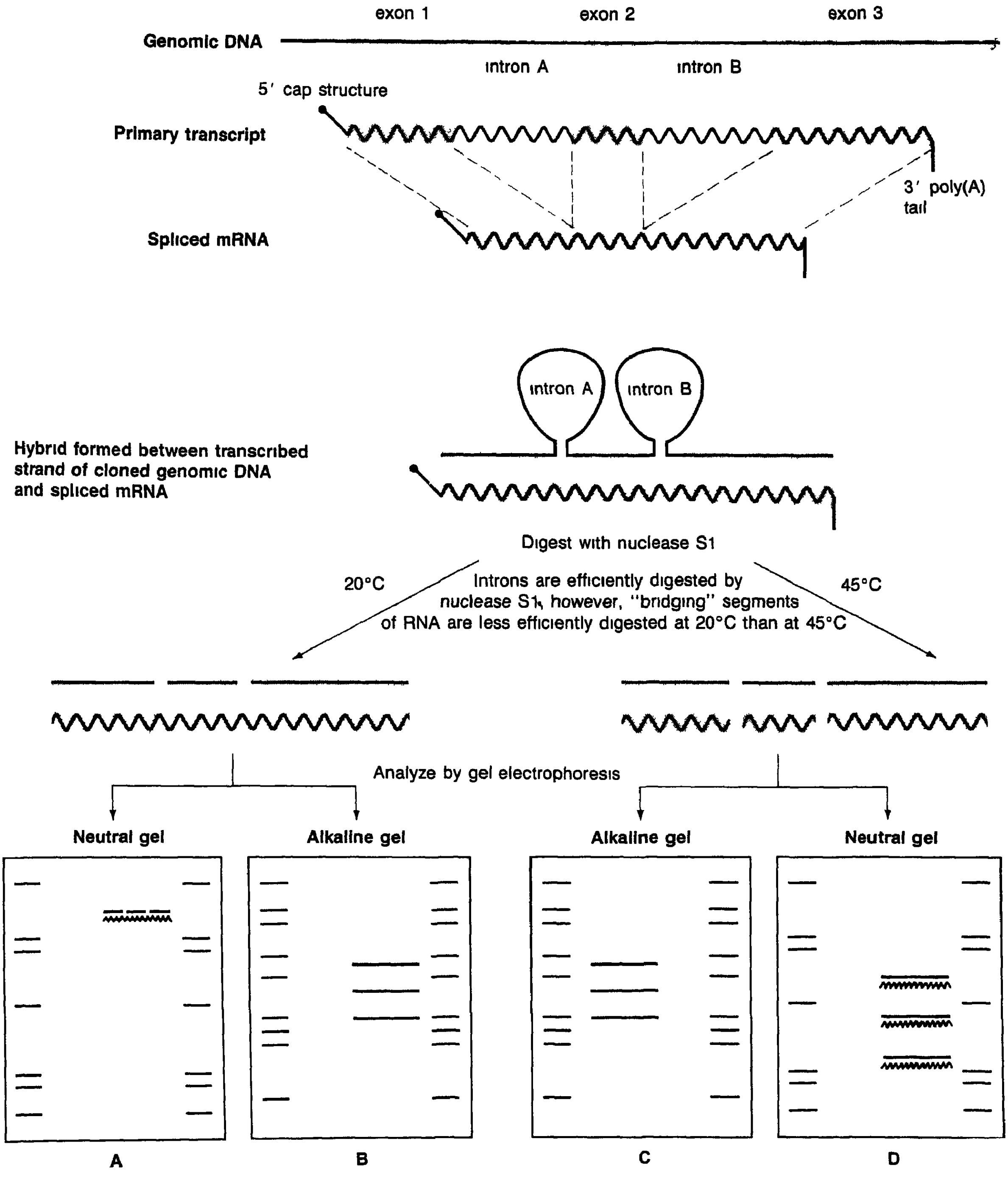
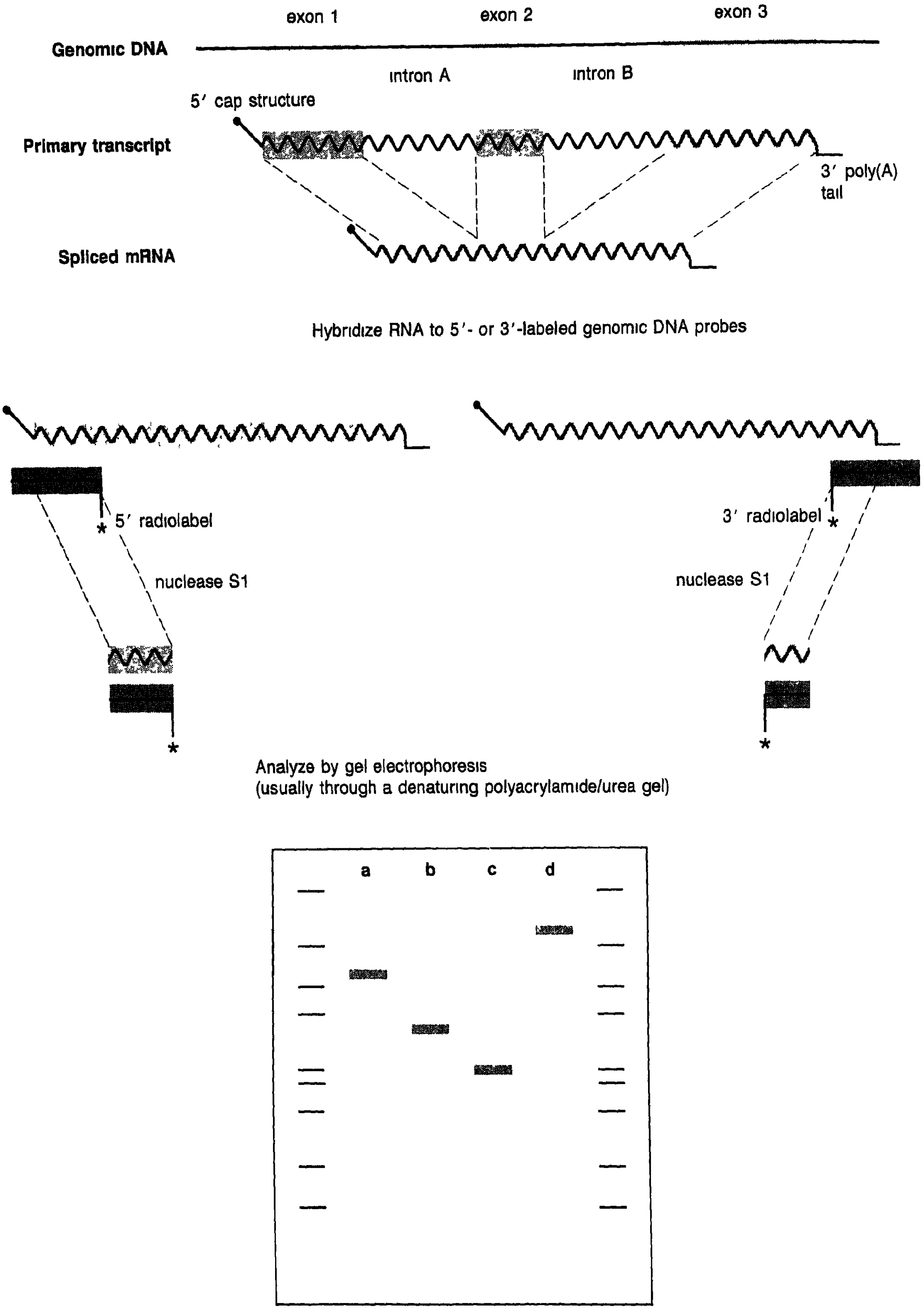

exon 1
exon 2
exon 3
Genomic DNA
intron A
intron B
5′ cap structure
Primary transcript
3′ poly(A) tail
Spliced mRNA
intron A
intron B
Hybrid formed between transcribed strand of cloned genomic DNA and spliced mRNA
Digest with nuclease S1
20°C
45°C
Introns are efficiently digested by nuclease S1; however, "bridging" segments of RNA are less efficiently digested at 20°C than at 45°C
Analyze by gel electrophoresis
Neutral gel
Alkaline gel
Alkaline gel
Neutral gel
A
B
C
D

FIGURE 7.3
Nuclease-S1 analysis of RNA Hybrids formed between the transcribed strand of genomic DNA and mRNA contain single-stranded loops of DNA (introns) Digestion of these hybrids with nuclease S1 at 20°C generates molecules whose RNA moieties are intact but whose DNAs contain gaps at the sites of the introns These molecules migrate as a single band when analyzed by gel electrophoresis under nondenaturing conditions (gel A) In alkaline gels (gel B), however, the RNA is hydrolyzed and the individual fragments of DNA separate according to their sizes When digestion is carried out at 45°C, both the DNA and RNA strands of the parental hybrid are cleaved to yield a series of smaller DNA RNA hybrids that can be separated by electrophoresis under nondenaturing conditions (gel D) The DNA moieties in these hybrids (gel C) are the same size as those detected in gel B



a = 5′-labeled probe before digestion with nuclease S1
b = 5′-labeled probe after digestion with nuclease S1
c = 3′-labeled probe after digestion with nuclease S1
d = 3′-labeled probe before digestion with nuclease S1

FIGURE 7.4 (See facing page for legend)

The first method given below describes the use of double-stranded DNA probes to map RNA with nuclease S1. The second, and more modern, method uses radiolabeled single-stranded probes. Whichever type of probe is used, it is important to realize that nuclease-S1 analysis of the structure of eukaryotic RNAs is not free of artifacts. For example, small mismatches in DNA RNA heteroduplexes are relatively resistant to the action of the nuclease (Berk and Sharp 1977). Conversely, regions of perfect heteroduplex that are rich in rU dA sequences are susceptible to cleavage (Miller and Sollner-Webb 1981) Third, a single molecule of DNA frequently can be protected from the action of the nuclease by simultaneous hybridization to two different RNA molecules (Lopata et al. 1985) Fourth, nuclease S1 cleaves the segment of DNA opposite a looped-out region of RNA comparatively inefficiently (Sisodia et al 1987). Although many of these problems can be solved by using a range of concentrations of nuclease S1, by performing the digestion at different temperatures, or by using a combination of nucleases (e g., RNAase H and nuclease S1) (Sisodia et al 1987), it is important to realize that cleavage by nuclease S1 of a DNA.RNA hybrid does not necessarily reflect a divergence in sequence between the two nucleic acids and that resistance to digestion is not necessarily synonymous with identity Mapping with nuclease S1 should therefore be regarded as a useful, but not infallible, guide to the structure of RNAs

FIGURE 7.4

Mapping the 5′ and 3′ termini of mRNAs Hybrids formed between mRNA and DNA probes radiolabeled at either their 5′ or their 3′ termini are digested with nuclease S1 By measuring the size of the nuclease-resistant fragments of DNA, it is possible to estimate the distance between the radiolabel and the 5′ and 3′ termini of the mRNA A similar strategy can be used to map the position of 3′ and 5′ splice sites

Mapping of RNA with Nuclease S1 and Double-stranded DNA Probes

1. Mix in a sterile microfuge tube.

DNA	0.1–1.0 μg
RNA	0.5–250 μg

The amount of DNA included in the reaction depends on its molecular weight For fragments approximately 5 kb in length, 1 μg of DNA is required, for smaller fragments, proportionately less DNA should be used

The amount of RNA required depends on the concentration of the sequences of interest Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of DNA and RNA that yield acceptable results To detect mRNA sequences present in low concentrations, up to 250 μg of RNA may be used in a 50-μl hybridization reaction For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less. If reagents are in short supply, the hybridization reaction can be scaled down to 10 μl

All reactions should contain the same amount of RNA to ensure that digestion with nuclease S1 is carried out under standard conditions If necessary, adjust the amount of RNA in the hybridization reactions by adding carrier RNA

Carrier RNA can be prepared by dissolving commercially available yeast tRNA at a concentration of 10 mg/ml in sterile TE (pH 7 6) containing 0 1 M NaCl The solution is then extracted twice with phenol (equilibrated in Tris Cl [pH 7 6] [see Appendix B]) and twice with chloroform, and the RNA is recovered by precipitation with 2 5 volumes of ethanol at room temperature The precipitated RNA is redissolved at a concentration of 10 mg/ml in sterile TE (pH 7 6), divided into small aliquots, and stored at −20°C

2. Precipitate the mixed DNA and RNA by adding 0 1 volume of 3 M sodium acetate (pH 5.2) and 2 5 volumes of ice-cold ethanol Store the solution at 0°C for at least 30 minutes Recover the nucleic acids by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge Discard the supernatant, wash the pellet with 70% ethanol, and recentrifuge Carefully remove all of the ethanol, and store the pellet of nucleic acids at room temperature until the last visible traces of ethanol have evaporated. Do not allow the pellet to become desiccated, otherwise it will be difficult to dissolve

3. Redissolve the pellet of nucleic acids in 30 μl of hybridization buffer Pipette the solution up and down many times to make sure that the pellet is completely dissolved.

It is often difficult to obtain complete dissolution in hybridization buffer of the pellet of nucleic acids that has been precipitated with ethanol This problem is exacerbated if the pellet is dried in a desiccator Sometimes the pellet can be redissolved by a combination of vigorous pipetting and heating to 60°C If difficulties persist, or if equivalent signals are not obtained from duplicate samples of RNA, the following procedure is recommended:

a After step 2, dissolve the pellet in 40–50 μl of water Evaporate the sample in a rotary evaporator until it is *just* dry
b. Add 30 μl of hybridization buffer The hydrated pellet goes into solution quickly and easily and gives extremely reproducible results.

Hybridization buffer

40 mM PIPES (pH 6.4)
1 mM EDTA (pH 8.0)
0.4 M NaCl
80% formamide

PIPES: Use the disodium salt of PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

4. Close the lid of the tube tightly, and incubate the hybridization reaction in a water bath set at 85°C for 10 minutes to denature the nucleic acids.

5. Rapidly transfer the tube to a water bath set at the desired hybridization temperature. Do not allow the tube to cool below the hybridization temperature during transfer. The hybridization temperature, which depends on the G + C content of the DNA, is chosen so as to minimize the formation of DNA·DNA hybrids while allowing DNA·RNA hybrids to form. Figure 7.5 shows the approximate hybridization temperatures for DNAs of different G + C content (Dean 1987). It is advisable to carry out a series of preliminary experiments to find out the optimal hybridization conditions for the RNA being used.

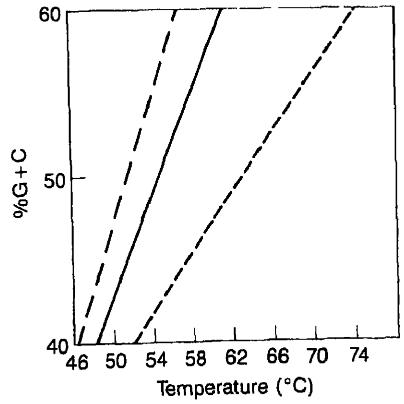


FIGURE 7.5
Optimization of yields of DNA·RNA hybrids. The graph (solid line) shows the temperature calculated to produce maximal yields of DNA·RNA hybrids when denatured DNA is annealed in the presence of RNA complementary to only one strand of the DNA. The broken lines show the calculated T_m for DNA.DNA (- - -) and DNA·RNA (---) hybrids. (Redrawn, with permission, from Dean 1987.)

6. Hybridize the DNA and RNA for 12–16 hours at the appropriate temperature. Then open the lid of the hybridization tube, taking care to keep the body of the tube submerged in the water. Rapidly add 300 μl of ice-cold nuclease-S1 mapping buffer, and immediately remove the tube from the water bath. Rapidly mix the contents of the tube by vortexing, and then transfer the tube to a water bath set at the temperature appropriate for digestion with nuclease S1 (see note below). Incubate for 1–2 hours, depending on the degree of digestion desired.

Nuclease-S1 mapping buffer

0.28 M NaCl
0.05 M sodium acetate (pH 4.5)
4.5 mM $ZnSO_4$
20 μg/ml single-stranded DNA (carrier DNA)
100–1000 units/ml nuclease S1

It is necessary to titrate the nuclease S1 each time a new probe or RNA is used.

Single-stranded DNA may be prepared by dissolving fragmented salmon sperm DNA in water at a concentration of 1 mg/ml. Add NaCl to a final concentration of 0.1 M, and extract the DNA solution with phenol:chloroform until no interface is visible. Recover the DNA from the aqueous phase by ethanol precipitation, and redissolve the DNA in a small volume of water. Adjust the concentration of the DNA solution to 1 mg/ml. Immerse the tube containing the DNA solution in a boiling-water bath for 20 minutes. Chill the DNA solution in ice water. Store the denatured DNA at 4°C.

A variety of temperatures and nuclease-S1 concentrations have been used to analyze DNA:RNA hybrids. At 20°C, nuclease S1 at a concentration of 100–1000 units/ml will degrade loops of DNA but will not efficiently digest segments of RNA molecules that bridge loops of DNA (see Figure 7.3). This can be an advantage when mapping intron/exon borders in segments of genomic DNA. The partially digested molecule shown in Figure 7.3 will migrate through agarose gels at neutral pH at approximately the same rate as double-stranded DNA; in alkaline conditions, the RNA "bridge" will be hydrolyzed, liberating two smaller pieces of single-stranded DNA. From the sizes of these fragments, it is often possible to assign locations to intron/exon junctions in the original segment of genomic DNA (Berk and Sharp 1977). For digestion of the single-stranded regions of DNA:RNA hybrids, higher temperatures (37°C–45°C) or increased quantities of nuclease S1 are generally required. However, it is rarely possible to establish conditions that will lead either to complete resistance to digestion or to complete digestion of structures of the type shown in Figure 7.3. The changing ratios of the various digestion products obtained under different conditions can often provide useful clues about the distribution of intron/exon borders in genomic DNA.

7. Chill the reaction to 0°C. Add 80 μl of nuclease-S1 stop mixture and mix.

Nuclease-S1 stop mixture

4 M ammonium acetate
50 mM EDTA (pH 8.0)
50 μg/ml tRNA (carrier RNA)

For preparation of carrier RNA, see notes to step 1, page 7.62.

8. Extract the reaction once with phenol:chloroform After centrifugation at 12,000*g* for 2 minutes at room temperature in a microfuge, transfer the aqueous supernatant to a fresh tube. Add 2 volumes of ethanol, mix, and store the tube at −20°C for 1 hour

9. Recover the nucleic acids by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge Carefully remove all of the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated

10. Dissolve the pellet in 40 μl of TE (pH 7 4)

11. Add 10 μl of loading buffer (50% glycerol and 0.2% bromocresol green), and mix well

12. Analyze the nuclease-S1-resistant hybrids by electrophoresis through an alkaline and/or a neutral agarose gel (see Chapter 6) As molecular-weight markers, use end-labeled fragments of DNA of known size

13. Following electrophoresis, transfer the DNA from either type of gel to a nitrocellulose filter or nylon membrane as described on pages 7 46–7.50. (Alkaline gels do not require soaking in denaturation solution)

14. Hybridize the filters to an appropriate ^{32}P-labeled DNA probe as described on page 7 52

Mapping of RNA with Nuclease S1 and Single-stranded DNA Probes

Single-stranded DNA probes are prepared by one of two methods.

- Separation of the strands of a fragment of double-stranded DNA
- De novo synthesis of a strand from a single-stranded template

Strand-separated probes are prepared by using restriction enzymes, singly or in combination, to generate a DNA fragment of suitable size (usually 100–500 nucleotides) with a 5′ extension at one end and a 3′ extension at the other. One strand of the fragment will therefore be up to 8 nucleotides longer than the other. This difference in size is sufficient to allow separation of the two strands by electrophoresis through a polyacrylamide gel under denaturing conditions Alternatively, if no convenient restriction sites are available, it is sometimes possible to purify the desired strand by agarose or polyacrylamide gel electrophoresis of denatured DNA under neutral conditions (Hayward 1972) (see Chapter 6) Either prior to or after electrophoresis, the 5′ terminus of the strand of interest is dephosphorylated with alkaline phosphatase and radiolabeled by addition of ^{32}P catalyzed by bacteriophage T4 polynucleotide kinase

De novo synthesis can be used to produce either end-labeled or uniformly labeled probes in vitro. In both cases, an oligonucleotide is used to prime the synthesis of a probe that is complementary to a single-stranded DNA template (usually derived from a recombinant bacteriophage M13) End-labeled probes are prepared by phosphorylating the 5′ terminus of the oligonucleotide primer; uniformly labeled probes are prepared by incorporating radiolabeled dNTPs into the growing strand. In both cases, the newly synthesized strand of DNA can be separated from the unlabeled template by digestion with a restriction enzyme that recognizes a unique site in the newly formed double-stranded DNA. The radiolabeled probe can then be separated from the linearized single-stranded DNA by electrophoresis through a polyacrylamide gel under denaturing conditions

Detailed descriptions of methods used to prepare and isolate radiolabeled single-stranded DNA probes are given in Chapter 10

1. Precipitate 0.5–150 μg of RNA by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. After storage at 0°C for 30 minutes, recover the RNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Rinse the pellet with 70% ethanol and recentrifuge. Carefully remove all of the ethanol, and store the pellet of nucleic acid at room temperature until the last visible traces of ethanol have evaporated

   The amount of RNA required depends on the concentration of the sequences of interest and on the specific activity of the radiolabeled DNA Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of DNA and RNA that yield acceptable results With DNAs that have been radiolabeled to high specific activity ($> 10^9$ cpm/μg), 10 μg of total RNA is usually sufficient to allow detection of mRNA species that are present at the level of 1–5 copies/cell To detect sequences present in lower concentrations (e g , in RNA extracted from heterogeneous populations of cells), up to 150 μg of RNA may be used in a 30-μl hybridization reaction For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less If reagents are in short supply, the hybridization reactions can be scaled down to 10 μl

   All reactions should contain the same amount of RNA to ensure that digestion with nuclease S1 is carried out under standard conditions If necessary, adjust the amount of RNA in the hybridization reactions by adding carrier RNA (For preparation of carrier RNA, see notes to step 1, page 7 62)

2. Redissolve the RNA in 30 μl of hybridization buffer. Pipette the solution up and down many times to make sure that the pellet is completely dissolved

   Hybridization buffer

   40 mM PIPES (pH 6.4)
   1 mM EDTA (pH 8.0)
   0.4 M NaCl
   80% formamide

   PIPES: Use the disodium salt of PIPES (piperazine-*N,N′*-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

   Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

   It is often difficult to obtain complete dissolution in hybridization buffer of RNA that has been precipitated with ethanol This problem is exacerbated if the pellet of RNA is dried in a desiccator Sometimes the RNA can be redissolved by a combination of vigorous pipetting and heating to 60°C If difficulties persist, or if equivalent signals are not obtained from duplicate samples of RNA, the following procedure is recommended:

a After step 1, dissolve the RNA in 40–50 μl of water Evaporate the sample in a rotary evaporator until it is *just* dry
b Add 30 μl of hybridization buffer The hydrated RNA goes into solution quickly and easily and gives extremely reproducible results

3. Add radiolabeled single-stranded DNA probe (~ 0 01 pmole—corresponding to ~1.6 ng of a fragment 400 nucleotides in length) The specific activity of the probe should be $> 5 \times 10^7$ cpm/μg, and it should be added in a volume of < 1 μl (see Chapter 10)

When using this method to measure the concentration of specific RNA sequences, it is essential to use an excess of radiolabeled probe This is usually determined empirically by nuclease-S1 digestion of a series of hybridization mixtures containing different ratios of RNA DNA If necessary, the system can be calibrated by setting up a series of control reactions containing a constant amount of radiolabeled single-stranded DNA and increasing amounts of RNA transcribed in vitro from an appropriate double-stranded DNA template (see Chapter 10)

Single-stranded probes uniformly labeled to high specific activity should be used within a few days to avoid problems caused by radiochemical degradation End-labeled probes can be stored for up to 1 week

4. Incubate the hybridization reaction at 85°C for 10 minutes to denature the nucleic acids, and then quickly transfer the reaction to a 30°C water bath. Incubate the hybridization reaction for 12–16 hours

5. Add to each reaction 300 μl of ice-cold nuclease-S1 mapping buffer. Mix well, and incubate for 1–2 hours at 12°C–45°C, depending on the expected structure of the DNA.RNA hybrid and the desired degree of digestion (see note below)

Nuclease-S1 mapping buffer

0.28 M NaCl
0.05 M sodium acetate (pH 4.5)
4.5 mM $ZnSO_4$
20 μg/ml single-stranded DNA (carrier DNA)
100–1000 units/ml nuclease S1

It is necessary to titrate the nuclease S1 each time a new probe or RNA is used

Single-stranded DNA may be prepared by dissolving fragmented salmon sperm DNA at a concentration of 1 mg/ml in water Add NaCl to a final concentration of 0 1 M, and extract the DNA solution with phenol chloroform until no interface is visible Recover the DNA from the aqueous phase by ethanol precipitation, and redissolve the DNA in a small volume of water Adjust the concentration of the DNA solution to 1 mg/ml Immerse the tube containing the DNA solution in a boiling-water bath for 20 minutes Chill the DNA solution in ice water Store the denatured DNA at 4°C

A variety of temperatures and nuclease-S1 concentrations have been used to analyze DNA RNA hybrids At 20°C, nuclease S1 at a concentration of 100–1000 units/ml will degrade loops of DNA but will not efficiently digest segments of RNA molecules that bridge loops of DNA (see Figure 7 3) This can be an advantage when mapping intron/exon borders in segments of genomic DNA The partially digested molecule shown in Figure 7 3 will migrate through agarose gels at neutral pH at approximately the same rate as double-stranded DNA, in alkaline conditions, the RNA "bridge" will be hydrolyzed, liberating two smaller pieces of single-stranded DNA From the sizes of these fragments, it is often possible to assign locations to intron/exon junctions in the original segment of genomic DNA (Berk and Sharp 1977) For digestion of the single-stranded regions of DNA RNA hybrids, higher temperatures (37°C–45°C) or increased quantities of nuclease S1 are generally required However, it is rarely possible to establish conditions that will lead either to complete resistance to digestion or to complete digestion of structures of the type shown in Figure 7 3 The changing ratios of the various digestion products obtained under different conditions can often provide useful clues about the distribution of intron/exon borders in genomic DNA

6. Chill the reaction to 0°C. Add 80 μl of nuclease-S1 stop mixture and mix.

Nuclease-S1 stop mixture

4 M ammonium acetate
50 mM EDTA (pH 8.0)
50 μg/ml tRNA (carrier RNA)

For preparation of carrier RNA, see notes to step 1, page 7 62

7. Recover the nucleic acids from the nuclease-S1 digest by precipitation with 2 volumes of ethanol. After storage for 60 minutes at −20°C, centrifuge the tube at 12,000*g* for 15 minutes at 4°C Carefully wash the pellet with 70% ethanol and recentrifuge. Remove the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated

8. Dissolve the pellet in 4 μl of TE (pH 7.4)

9. Add 6 μl of formamide loading buffer and mix well.

Formamide loading buffer

80% formamide
10 mM EDTA (pH 8.0)
1 mg/ml xylene cyanol FF
1 mg/ml bromophenol blue

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized as described in step 2, page 7.67.

10. Heat the nucleic acids for 5 minutes at 95°C, and then immediately transfer the tube to an ice bath

11. Analyze the radiolabeled DNA by electrophoresis through a polyacrylamide/7 M urea gel (see Chapter 13) cast according to the expected sizes of the radioactive fragments As molecular-weight markers, use end-labeled fragments of DNA of known size

% Polyacrylamide/urea gel	Size of band (nucleotides)
4	>250
6	60–250
8	40–120
10	20–60
12	10–50

Tracking dyes migrate through denaturing polyacrylamide gels at the following approximate positions equivalent to nucleotide migration

% Polyacrylamide/urea gel	Xylene cyanol FF (nucleotides)	Bromophenol blue (nucleotides)
4	155	30
6	110	25
8	75	20
10	55	10

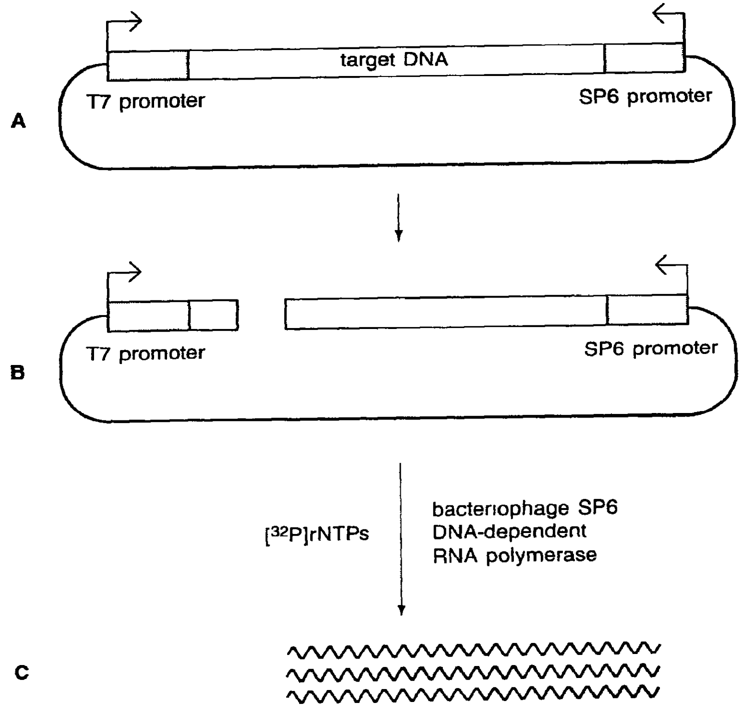
12. Establish an autoradiographic image of the gel as described in Appendix E

MAPPING OF RNA WITH RIBONUCLEASE AND RADIOLABELED RNA PROBES

Radiolabeled RNA probes can be used in hybridization experiments in the same way as single-stranded DNA probes to detect and quantitate specific RNAs and to study gene and mRNA structure (Lynn et al. 1983; Zinn et al. 1983; Cox et al. 1984) The primary differences between the two procedures are the methods used to synthesize the probes and the use of RNAase to digest regions of the probe that do not form hybrids with the corresponding gene or mRNA (Zinn et al. 1983).

The strategy for mapping mRNA with radiolabeled RNA probes generated in vitro is illustrated in Figure 7 6 A segment of DNA containing all or part of the gene of interest is inserted into a polycloning site immediately downstream from a bacteriophage T7 or SP6 promoter, in an orientation that leads to the production of antisense RNA (Zinn et al 1983; Melton et al. 1984) The recombinant plasmid is digested with a restriction enzyme that cleaves at a convenient site within the gene or at a site in the plasmid downstream from it. The linearized plasmid is then transcribed in the presence of ^{32}P-labeled rNTPs with the appropriate bacteriophage DNA-dependent RNA polymerase to produce an RNA that extends from the initiation site of the promoter to the end of the DNA fragment. An excess of this ^{32}P-labeled single-stranded RNA is hybridized in solution with the RNA being tested so that all complementary sequences are driven into ^{32}P-labeled RNA:RNA hybrids The amount of radiolabeled RNA required to force hybridization to completion is usually determined empirically in preliminary experiments After unhybridized material has been removed by digestion with RNAase, the radiolabeled RNA·RNA hybrid is then detected and quantitated by polyacrylamide gel electrophoresis under denaturing conditions, followed by autoradiography

Mapping of RNA with RNAase is extremely sensitive: Reconstruction experiments have shown that as little as 0 1 pg of mRNA can be detected by exposing the autoradiograph for several days (Melton et al. 1984). The sensitivity of this method is therefore approximately 20-fold greater than that attainable with double-stranded DNA probes or end-labeled single-stranded DNA probes and approximately equal to that attainable with uniformly labeled single-stranded DNA probes Furthermore, the digestion of RNA RNA hybrids with RNAase appears to suffer from fewer artifacts than digestion of RNA.DNA hybrids with nuclease S1 For these reasons and because of the relative ease with which radiolabeled RNA probes can be synthesized, it is not surprising that RNAase digestion of RNA:RNA hybrids has become a standard method to quantitate mRNA molecules, to map their termini, and to determine the positions of introns within the corresponding gene.



Clone target DNA into a plasmid downstream from a prokaryotic promoter (bacteriophage SP6 or T7)

Linearize recombinant plasmid with restriction enzyme that cleaves at distal end of target DNA

Transcribe linear DNA in vitro with appropriate DNA-dependent RNA polymerase and remove template DNA by digestion with pancreatic DNAase I

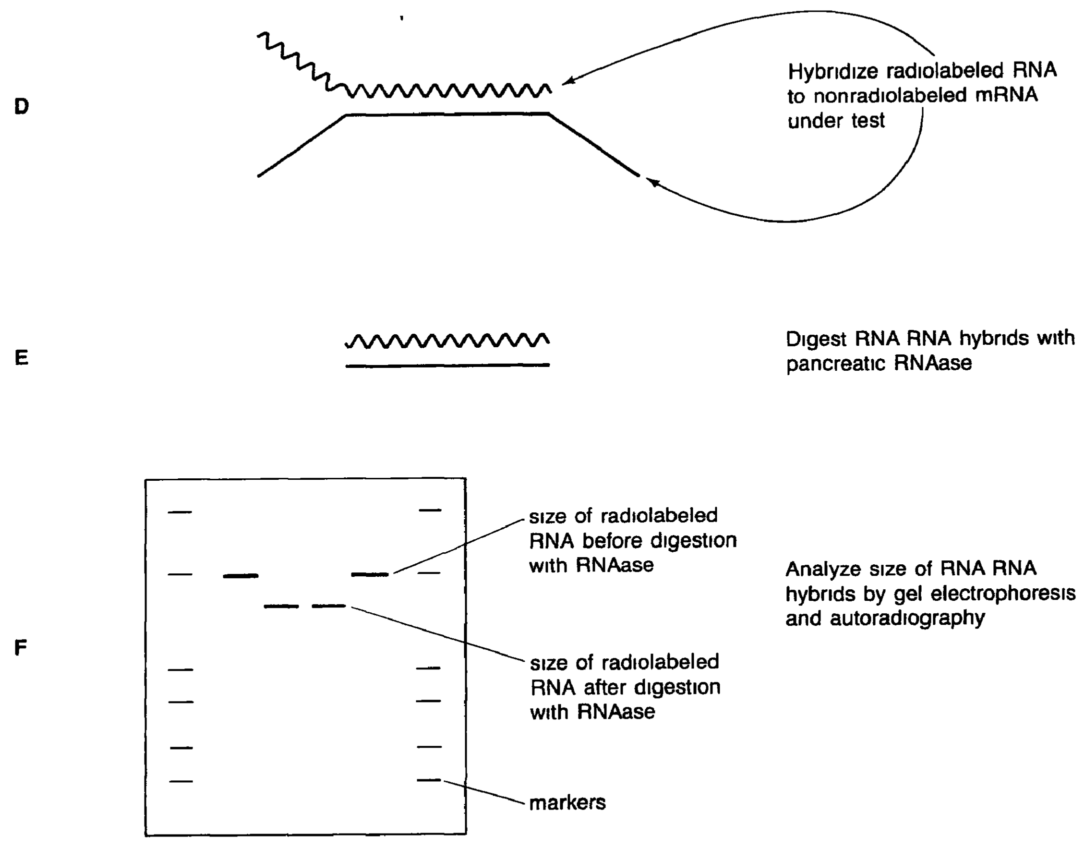

D
Hybridize radiolabeled RNA to nonradiolabeled mRNA under test
E
Digest RNA RNA hybrids with pancreatic RNAase
F
size of radiolabeled RNA before digestion with RNAase
size of radiolabeled RNA after digestion with RNAase
markers
Analyze size of RNA RNA hybrids by gel electrophoresis and autoradiography

FIGURE 7.6
Mapping of mRNAs with radiolabeled RNA probes Radiolabeled RNA, synthesized in vitro from a cloned copy of the template DNA (steps A–C), is hybridized to unlabeled test mRNA (step D) After removal of the unhybridized tails (step E), the size of the radiolabeled RNA that is resistant to RNAase is determined by gel electrophoresis (step F) By using probes complementary to appropriate segments of the template DNA, it is possible to map the positions of the 5′ and 3′ termini of mRNAs and the positions of 5′ and 3′ splice sites

1. Synthesize a uniformly labeled, strand-specific RNA probe as described in Chapter 10. The probe should be used within a few days to avoid problems caused by radiochemical damage to the RNA.

2. Precipitate 0.5–150 μg of the RNA that is to be analyzed by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. Store the solution at 0°C for 30 minutes, and then recover the RNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol and recentrifuge. Carefully remove all of the ethanol, and store the pellet at room temperature until the last visible traces of ethanol have evaporated.

   The amount of unlabeled RNA required depends on the concentration of the sequences of interest and the specific activity of the radiolabeled complementary RNA. Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of RNAs that yield acceptable results. With RNA probes that have been radiolabeled to high specific activity ($>10^9$ cpm/μg), 10 μg of total RNA is usually sufficient to allow detection of mRNA species that are present at the level of 1–5 copies/cell. To detect sequences present in lower amounts (e.g., in RNA extracted from heterogeneous populations of cells), up to 150 μg of RNA may be used in a 30-μl hybridization reaction. For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less. If reagents are in short supply, the hybridization reactions can be scaled down to 10 μl.

   When comparing different preparations of RNA, make sure that all of the reactions contain the same amount of RNA. In this way, digestion with RNAase is carried out under standard conditions. If necessary, adjust the amount of RNA in the hybridization reactions by adding carrier RNA. (For preparation of carrier RNA, see notes to step 1, page 7.62.)

3. Redissolve the RNA to be analyzed in 30 μl of hybridization buffer containing a small molar excess of radiolabeled probe. Pipette the solution up and down many times to make sure that the pellet is completely dissolved.

   Hybridization buffer

   40 mM PIPES (pH 6.4)
   1 mM EDTA (pH 8.0)
   0.4 M NaCl
   80% formamide

   PIPES. Use the disodium salt of PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

   Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

It is often difficult to obtain complete dissolution in hybridization buffer of RNA that has been precipitated with ethanol The problem is exacerbated if the pellet is dried in a desiccator Sometimes the RNA can be redissolved by a combination of vigorous pipetting and heating to 60°C If difficulties persist, or if equivalent signals are not obtained from duplicate samples of RNA, the following procedure is recommended

a After step 2, dissolve the RNA in 40–50 μl of water Evaporate the sample in a rotary evaporator until it is *just* dry

b Add 30 μl of hybridization buffer The hydrated RNA goes into solution quickly and easily and gives extremely reproducible results

Usually, 1×10^5 to 5×10^5 cpm of probe are used per hybridization A single in vitro transcription reaction with bacteriophage SP6 or bacteriophage T7 DNA-dependent RNA polymerase generates enough probe (~2 pmoles) for more than 200 hybridization reactions However, because background increases as more probe is added to the hybridization reaction, add only enough probe to achieve a small molar excess This is usually determined empirically by RNAase digestion of a series of hybridization mixtures containing different ratios of probe RNA to target RNA If necessary, the system can be calibrated by setting up a series of control reactions containing a constant amount of radiolabeled RNA and increasing amounts of unlabeled RNA transcribed in vitro from the opposite strand of an appropriate double-stranded DNA template (see Chapter 10)

4. Incubate the hybridization mixture at 85°C for 10 minutes to denature the RNAs. Quickly transfer the hybridization mixture to a water bath set at the annealing temperature Incubate the mixture for 8–12 hours.

The optimal temperature for annealing varies from RNA to RNA, presumably depending on such factors as G + C content and propensity to form secondary structures In most cases, satisfactory results are obtained when the RNA is annealed at 45°C–50°C Optimal conditions for the hybridization of specific probes can be established by reconstruction experiments, in which the radiolabeled probe is hybridized at temperatures ranging between 25°C and 65°C to unlabeled RNA transcribed in vitro from the opposite strand of an appropriate double-stranded DNA template (see Chapter 10)

5. Cool the hybridization mixture to room temperature, and add 300 μl of RNAase digestion mixture

RNAase digestion mixture

300 mM NaCl
10 mM Tris · Cl (pH 7.4)
5 mM EDTA (pH 7.5)
2 μg/ml RNAase T1
40 μg/ml RNAase A

Digest the hybridization reaction for 60 minutes at 30°C

Concentrated stock solutions of RNAases are usually made up in 10 mM Tris Cl (pH 7 5), 15 mM NaCl and stored at −20°C (RNAase A, 10 mg/ml [see Appendix B], RNAase T1, 100 μg/ml)

The time and temperature of the digestion with RNAase should be determined empirically if the signal-to-noise ratio is unacceptable

6. Add 20 μl of 10% SDS and 10 μl of a 10 mg/ml solution of proteinase K. Incubate the reaction for 30 minutes at 37°C

The solution of proteinase K should be freshly prepared Solutions that have been frozen or thawed several times do not work well and sometimes lead to the degradation of RNA

7. Add 400 μl of phenol:chloroform, vortex for 30 seconds, and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge

8. Transfer the upper, aqueous phase to a fresh tube, carefully avoiding the interface between the organic and aqueous phases

9. Add 20 μg of tRNA and 750 μl of ice-cold ethanol Mix well by vortexing, and store the solution at −20°C for 30 minutes

10. Recover the RNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge Carefully remove the ethanol and wash the pellet with 500 μl of 70% ethanol Recentrifuge

11. Carefully remove all of the ethanol, and store the open tube at room temperature until the last visible traces of ethanol have evaporated

12. Resuspend the precipitate in 10 μl of formamide loading buffer

Formamide loading buffer

80% formamide
10 mM EDTA (pH 8.0)
1 mg/ml xylene cyanol FF
1 mg/ml bromophenol blue

Formamide. Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment However, if any yellow color is present, the formamide should be deionized as described in step 3, page 7.74

13. Heat the nucleic acids for 5 minutes at 95°C, and then immediately transfer the tube to an ice bath

14. Analyze the radiolabeled RNA by electrophoresis through a polyacrylamide/7 M urea gel (see Chapter 13) cast according to the expected size of the radioactive fragments (see step 11, page 7 70). As molecular-weight markers, use end-labeled fragments of DNA of known size

The relative mobility of RNA and DNA through polyacrylamide/7 M urea gels varies according to the conditions used for electrophoresis In general, the faster the gel is run, the less the difference in mobility between RNA and DNA molecules of identical size. Under the conditions normally used (40–45 V/cm), RNA runs approximately 5%–10% slower than DNA of the same size Thus, a 90-nucleotide RNA will migrate at approximately the same rate as a 100-nucleotide DNA. If absolute measurement of the size of the RNA is required, a series of radioactive probes of defined length can be generated by in vitro transcription of a double-stranded DNA template after digestion with restriction enzymes that cleave at sites progressively more distal to a bacteriophage promoter or by end-labeling of commercially available RNA markers

15. Establish an autoradiographic image of the gel as described in Appendix E

Notes

i. It is important to remove the template DNA completely from the transcription reaction by digestion with RNAase-free pancreatic DNAase I (see Chapter 10) Trace amounts of partially degraded template DNA in the hybridization mixture will lead to the formation of DNA:RNA hybrids that will be detected on the autoradiograph. This problem can be minimized by titrating the amount of probe needed for each test sample to give a strong signal without a large amount of background.

ii. A control hybridization reaction containing 20 μg of tRNA instead of the RNA being tested should always be carried out to check for spurious protection of the probe from digestion by RNAases RNAase-resistant fragments can be formed if the probe contains self-complementary sequences or if it is contaminated with a small quantity of RNA transcribed from the opposite strand of the DNA template. These rare complementary transcripts are thought to be generated by transcription initiated by bacteriophage T7 and SP6 RNA polymerases at the ends of the template (Schenborn and Mierendorf 1985) Significant quantities of these extraneous transcripts are synthesized in vitro only when the linearized DNA templates carry protruding 3′ termini. This problem can therefore be minimized by using a restriction enzyme that generates 5′ protruding termini to linearize the plasmid or by modifying the termini with bacteriophage T4 DNA polymerase before the transcription reaction If all other procedures fail, it is possible to purify a probe of the desired length by agarose or polyacrylamide gel electrophoresis.

iii Problems occasionally arise because of degradation of the probe before the hybridization reaction or, more rarely, because of the synthesis of transcripts that are less than full-length. In both cases, many smaller fragments are observed in addition to a protected RNA fragment of the expected size. Check that none of the reaction buffers or enzymes is contaminated with RNAase, and analyze an aliquot of the probe by electrophoresis through a polyacrylamide gel under denaturing conditions In a typical reaction with bacteriophage SP6 or T7 RNA polymerase, more than 90% of the transcripts will appear as a single band of the expected size Contamination with RNAase will result in the appearance of a smear of smaller fragments of RNA in the gel. This problem can be circumvented by making fresh buffers and checking them for RNAase contamination using the gel electrophoresis assay. Transcripts that are less than full-length may also be generated if the conditions for transcription are not optimal (see Melton et al. 1984; Krieg and Melton 1987) or if there are signals for termination of transcription in the template. Once again, this problem can be solved by purifying the transcripts of the correct length by agarose or polyacrylamide gel electrophoresis.

iv. Certain batches of ^{32}P-labeled UTP contain inhibitors that can cause premature termination of nascent transcripts This problem can usually be circumvented by using another radiolabeled nucleotide

ANALYSIS OF RNA BY PRIMER EXTENSION

Primer extension is used to map and quantitate the 5′ termini of RNA and to detect precursors and processing intermediates of mRNA. The test RNA is hybridized with an excess of a single-stranded DNA primer (a synthetic oligonucleotide or a restriction fragment) radiolabeled at its 5′ terminus. Reverse transcriptase is then used to extend this primer to produce cDNA complementary to the RNA template The length of the resulting end-labeled cDNA, as measured by electrophoresis through a polyacrylamide gel under denaturing conditions, reflects the distance between the end-labeled nucleotide of the primer and the 5′ terminus of the RNA. The yield of cDNA is approximately proportional to the concentration of the target sequences in the mRNA preparation.

If desired, the end-labeled cDNA can be extracted from the gel and sequenced by the Maxam-Gilbert method. It is also possible to sequence the cDNA directly by including dideoxyribonucleoside triphosphates in the primer-extension reaction, but this should be done only after it has been established that a single species of cDNA is synthesized during the reaction.

Either single-stranded or denatured double-stranded DNAs can be used as primers In the latter case, the conditions for hybridization are adjusted to suppress the formation of DNA.DNA hybrids in favor of DNA:RNA hybrids. Because it is not usually possible to prevent completely the formation of DNA DNA hybrids, it is advisable always to include controls that contain no RNA in the hybridization reaction. Fragments of double-stranded DNA used as primers are usually 75–150 nucleotides in length and are radiolabeled at both ends by phosphorylation or by incorporation of radiolabeled dNTPs at a recessed 3′ terminus

Single-stranded DNA primers, which are preferred for experiments of this type, are synthetic oligonucleotides 30–40 nucleotides in length Primers of this type have two main advantages· They eliminate the formation of DNA DNA hybrids and they can be precisely designed to hybridize to specific sequences of the mRNA In this way, it is possible to avoid potential problems caused by secondary structure in the mRNA and to maximize resolution of the cDNA products from the primer by gel electrophoresis.

It is important to use primers whose target sequences are located within 100 nucleotides of the 5′ terminus of the mRNA Primers that hybridize to more-distal sites frequently generate heterogeneous extension products because of the tendency of reverse transcriptase to stop or pause in regions of high secondary structure in the template RNA

1. Radiolabel the fragment of DNA to be used as a primer. Double-stranded DNA may be labeled either by phosphorylation or by filling of a recessed 3′ terminus (see Chapter 10). Oligonucleotides are labeled by phosphorylation (see Chapter 11).

   The length of the primer is not critical and can range from 75 to 150 or more nucleotides. However, the shorter the primer, the greater the difference in size between it and the extended product.

2. Mix 10^4–10^5 cpm of the DNA primer with 0.5–150 μg of the RNA that is to be analyzed. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. Store the solution at −20°C for 30 minutes, and then recover the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol and recentrifuge. Carefully remove all of the ethanol, and store the pellet at room temperature until the last visible traces of ethanol have evaporated.

   The amount of unlabeled RNA required depends on the concentration of the sequences of interest. Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of RNAs that yield acceptable results. Usually, 10 μg of total RNA is sufficient to allow mapping of the 5′ termini of mRNA species that are present at the level of 1–5 copies/cell. To analyze RNAs present in lower amounts (e.g., in RNA extracted from heterogeneous populations of cells), up to 150 μg of RNA may be used in a 30-μl hybridization reaction. For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less. If reagents are in short supply, the hybridization reactions can be scaled down to 10 μl.

   To minimize problems caused by contamination with RNAase, treat all of the reagents and tubes as described on pages 7.3–7.4.

3. Redissolve the precipitated nucleic acids in 30 μl of hybridization buffer. Pipette the solution up and down many times to make sure that the pellet is completely dissolved.

   Hybridization buffer

   40 mM PIPES (pH 6.4)
   1 mM EDTA (pH 8.0)
   0.4 M NaCl
   80% formamide

   PIPES. Use the disodium salt of PIPES (piperazine-*N,N*′-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

   Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

It is often difficult to obtain complete dissolution in hybridization buffer of the pellet of nucleic acids that has been precipitated with ethanol This problem is exacerbated if the pellet is dried in a desiccator Sometimes the pellet can be redissolved by a combination of vigorous pipetting and heating to 60°C If difficulties persist, or if variable signals are obtained from duplicate samples of RNA, the following procedure is recommended

a After step 2, dissolve the pellet in 40–50 μl of water. Evaporate the sample in a rotary evaporator until it is *just* dry
b Add 30 μl of hybridization buffer The hydrated pellet goes into solution quickly and easily and gives extremely reproducible results

Usually, 10^4–10^5 cpm of primer are used per hybridization However, because the number of artifactual bands increases as more primer is added to the hybridization reaction, it is advisable to carry out a series of pilot reactions that contain a constant amount of RNA and different amounts of primer

4. Incubate the hybridization mixture at 85°C for 10 minutes to denature the nucleic acids. Quickly transfer the hybridization mixture to a water bath set at the annealing temperature Incubate the mixture for 8–12 hours

The optimal temperature for annealing varies from RNA to RNA, presumably depending on such factors as G + C content, its propensity to form secondary structures, and the length of the primer Optimal conditions should be established in a series of pilot experiments With double-stranded DNA primers, the aim is to minimize formation of DNA.DNA hybrids and to maximize annealing of the primer to its target RNA In most cases, satisfactory results are obtained at 40°C–50°C With oligonucleotide primers, the aim is to minimize formation of mismatched hybrids Primers 30–40 nucleotides in length usually yield satisfactory results when annealed to RNA at 30°C

5. Add 170 μl of water and 400 μl of ethanol. Mix well, and store at 0°C for 1 hour Collect the precipitate of nucleic acids by centrifugation at 12,000*g* for 15 minutes at 0°C in a microfuge Carefully discard the supernatant and wash the pellet with 500 μl of 70% ethanol Recentrifuge Carefully discard the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.

6. Redissolve the primer.RNA hybrids in 20 μl of reverse transcriptase buffer

Reverse transcriptase buffer

50 mM Tris · Cl (pH 7.6)
60 mM KCl
10 mM $MgCl_2$
1 mM of each dNTP
1 mM dithiothreitol
1 unit/μl placental RNAase inhibitor
50 μg/ml actinomycin D

Add 50 units of murine reverse transcriptase, and mix well by gentle vortexing. Try to avoid making bubbles. This is sometimes difficult because reverse transcriptase is supplied in a buffer containing Triton X-100. Bubbles may be removed by brief centrifugation in a microfuge. Incubate the reaction for 2 hours at 37°C.

Once initiated by a primer, synthesis of a cDNA molecule usually proceeds to the 5′ terminus of the RNA template. However, regions of the template that are rich in secondary structure may cause reverse transcriptase to pause or stop, resulting in cDNAs that are less than full-length. This artifact can be minimized by performing the reaction in the presence of higher (5 mM) concentrations of dNTPs and using a primer that is complementary to sequences of the mRNA that lie within 50–100 nucleotides of its 5′ terminus. However, to distinguish between artifacts of reverse transcription and true heterogeneity of 5′ termini, it is often necessary to carry out nuclease-S1 analysis with probes labeled at their 5′ termini.

Actinomycin D inhibits synthesis of double-stranded DNA by reverse transcriptase without significantly affecting the yield of first-strand DNA. It therefore prevents the synthesis of "hairpin" molecules that are formed when the first strand of DNA serves as a primer/template. The efficiency of this self-priming is believed to vary widely from mRNA to mRNA but is generally quite low. However, since actinomycin D does not affect the efficiency with which mRNA is copied into DNA, its routine use is recommended to reduce the possibility of obtaining artifactual products of the primer-extension reaction. Stock solutions of actinomycin D are usually prepared in ethanol at a concentration of 5 mg/ml, stored at -20°C in the dark, and diluted into the reaction mixture immediately before use.

Caution: Actinomycin D is a teratogen and a carcinogen. Stock solutions should be prepared, wearing gloves and a mask, in a chemical hood, not on an open bench. Solutions of actinomycin D are light-sensitive.

7. Add 1 μl of 0.5 M EDTA (pH 8.0) and 1 μl of DNAase-free pancreatic RNAase (5 μg/ml) (see Appendix B). Incubate the reaction for 30 minutes at 37°C.

8. Add 150 μl of TE (pH 7.6), containing 0.1 M NaCl, and 200 μl of phenol:chloroform. Vortex for 30 seconds, and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge.

9. Transfer the upper, aqueous phase to a fresh tube, carefully avoiding the interface between the organic and aqueous phases.

10. Add 500 μl of ethanol. Mix well, and store at 0°C for 1 hour.

11. Recover the nucleic acids by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Carefully remove the ethanol and wash the pellet with 500 μl of 70% ethanol. Recentrifuge.

12. Carefully remove all of the ethanol, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.

13. Dissolve the pellet in 4 μl of TE (pH 7.4), add 6 μl of formamide loading buffer, and mix well.

Formamide loading buffer

80% formamide
10 mM EDTA (pH 8.0)
1 mg/ml xylene cyanol FF
1 mg/ml bromophenol blue

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized as described in step 3, page 7.80.

14. Heat the mixture for 5 minutes at 95°C, and immediately transfer the tube to an ice bath

15. Analyze the radiolabeled DNA by electrophoresis through a polyacrylamide/7 M urea gel (see Chapter 13) cast according to the expected sizes of the radioactive fragments (see step 11, page 7.70). As molecular-weight markers, use end-labeled fragments of DNA of known size.

16. Establish an autoradiographic image of the gel as described in Appendix E

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